Microbial inoculants: Global reliability and specific application in a mixed cropping system on marginal land in India

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Abbreviations

AMF – Arbuscular mycorrhizal fungi
ARISA – Automated ribosomal intergenic spacer analysis
CFU – Colony forming unit
DNA – Deoxyribonucleic acid
FC – Field capacity
FM – Finger millet
FYM – Farm yard manure
HC – Hyphal compartment
HLD – Hyphal length density
ICP-OES - Inductively coupled plasma atomic emission spectroscopy
ITS – Internal transcribed spacer
OTU – Operational taxonomic unit
PCR – Polymerase chain reaction
PGPR – Plant growth promoting rhizobacteria
PP – Pigeon pea
UV/vis spectroscopy - Ultraviolet–visible spectroscopy

Contents

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Summary

The application of microbial inoculants (biofertilizers) is a promising technology for future sustainable farming systems in view of rapidly decreasing phosphate stocks and the need to more efficiently use available nitrogen (N). Various microbial taxa are currently used as biofertilizers, based on their capacity to access nutrients from fertilizers and soil stocks, to fix atmospheric nitrogen, to improve water uptake or to act as biocontrol agents. Since the results of biofertilization in the field are inconsistent we conducted a meta-analysis to quantify benefits of biofertilizers in terms of yield increase, nitrogen and phosphorus use efficiency, based on 171 peer reviewed publications that met the eligibility criteria. Major findings are: i) the superiority of biofertilizer performance in dry climates over other climatic regions; ii) yield response due to biofertilizer application was generally small at low soil P levels; efficacy increased along higher soil P levels in the order arbuscular mycorrhizal fungi (AMF), P-solubilizers and N-fixers; iii) success of inoculation with AMF was greater at low organic matter content and at neutral pH. Our comprehensive analysis provides a basis and guidance for proper choice and application of biofertilizers.

Rainfed farms on marginal lands will be most affected by scarcity of non-renewable resources such as fertilizers. Mutualistic root organisms like AMF can substantially contribute to a more resilient, sustainably intensified dryland farming system. We are interested to study the possibility to use AMF as "biofertilizers" in an intercropping system in Indian agriculture, planting pigeon pea (*Cajanus cajan*) seedlings pre-inoculated with AMF into a field sown with finger millet (*Eleusine coracana*). By destructive sampling over five weeks we estimated a hyphal growth of 4.1mm d^{-1} by *C. etunicatum* which is 1mm faster per day than all other estimates. To study the potential of *Rhizophagus fasciculatus, Claroideoglomus etunicatum* and *Rhizophagus intraradices* to spread from AMF-inoculated pigeon pea to un-inoculated finger millet seedlings, we established experimental microcosms in the greenhouse, in which the pigeon pea and two finger millet plantlets were kept in separate pots, connected by soil bridges of 5 or 12 cm length inaccessible to roots but accessible to fungal hyphae. We found that depending on the distance different AMF were promoting the growth of finger millet better. We also detected transport of fertilized nitrogen along the hyphae via stable isotope analysis over a distance of up to 12 cm. However these results also depended on the AMF species. We conclude that the row distance between the crops and the choice of AMF species play a crucial role for the application of AMF as biofertilizer and their growth promotion.

To understand the effects the biofertilizers *Pseudomonas fluorescens* and two AMF species on the microbial community in the soil, both the bacterial community and the community of AMF were studied. Samples were collected at harvest from mono- and intercropped pigeon pea and finger millet at two field sites in South India at the University of Agricultural Sciences, GKVK campus, Bangalore and Kolli hills, Tamil Nadu state, India. DNA was extracted from rhizosphere soil surrounding the roots. To detect changes in the bacterial community automated ribosomal intergenic spacer analysis (ARISA) was conducted and treatments were compared using principal component analysis. The strongest effect was found to be exerted by the plant species; biofertilization had no effect on the bacterial community. To detect changes in the AMF community we amplified the whole ITS ribosomal unit and sequenced the barcoded samples with the PacBio platform. Although OTUs from Glomeromycota were found, the sequencing depth remained too little to make firm conclusions about the changes in the AMF community. Our second goal was to trace the applied inoculum at harvest and, although only few sequences were recovered, the inoculum of *Rhizophagus fasciculatus* could be traced in some treatments.

Introduction

The current rate of decline of the earth's natural resources, particularly of the reserves of rock phosphate and fossil fuel, is of great concern for the future of agriculture. Agriculture is the main consumer of phosphorus in the form of mineral fertilizers. Phosphate fertilizers are mined from only a few rock phosphate deposits in the world and the peak of extraction of phosphate is expected to happen in the 2030s (Cordell et al. 2009) with the prices expected to increase afterwards due to the higher extraction cost. Fossil fuels are needed to produce nitrogen fertilizers, highly needed for industrial agriculture, by fixing atmospheric nitrogen to ammonia in the Haber-Bosch process. Besides the decline of fossil fuels, the use of fossil fuels enriches the atmosphere with fossil carbon dioxide. This increase of carbon dioxide has been recognized as the source of climate to change during the 20^{th} century also known as anthropogenic climate change (Karl and Trenberth 2003). This global problem has greater implications for developing countries especially in the tropics because weathered soils with nutrient deficiencies and ion toxicities are more common there and then rely more on external inputs to keep up food production. Furthermore people are more food insecure (Clair and Lynch 2010).

Efforts to mitigate the declining mineral nutrient reserves are currently major topics of research but the perturbance of the global biogeochemical cycles, mainly driven by the use of mineral fertilizers, remains a serious problem (Kahiluoto et al. 2014). Nutrients in intensive agriculture are only used in part by the crop, another part remains in the soil; but the main driver of this perturbance is the part that is lost from the ecosystem, by erosion in case of phosphate, or by volatilization or leaching in case of nitrogen. When nitrogen fertilizers are applied, the N often enters the soil ecosystem in the form of nitrate. Under low oxygen concentrations in the soil some of the nitrate is denitrified by bacteria which results in N_2 and nitrous oxide and is then lost for crop production. Nitrous oxide is particularly problematic since it is a "greenhouse gas", and its release causes climate change to accelerate (Mosier et al. 1998). Another part of nitrate is lost by rainfall which transports and leaches it to the groundwater. As a side effect lakes or even the sea are "fertilized" causing algal blooms and their subsequent death causes so-called dead zones without any oxygen, important for any lifeforms, in these water bodies even in large ones like the Baltic Sea or the Gulf of Mexico (Rabalais et al. 2002).

Sustainable crop production has to reduce the perturbance of the nutrient cycles and find ways to increase the use efficiency of fertilizers by at the same time protecting biodiversity and soils. Practices to achieve this have different names like agroecology, organic agriculture or sustainable agriculture, but all try the same. Sustainable crop production remains a major global

challenge and has drawn increasing attention among policy makers, business and the scientific community (Wezel et al. 2014).

What are biofertilizers?

Microbial inoculants, so-called biofertilizers, are a promising technology to reduce the use of conventional inorganic fertilizers. Rhizosphere microorganisms are either growing in the rhizosphere or as endophytes inside the roots. Many of them can serve as biofertilizers as they are able to fix nitrogen (N), help to access nutrients such as phosphorus (P) and N from organic fertilizers and soil stocks, improve drought tolerance, improve plant health or increase salt tolerance.

The discovery of Rhizobia as the first commercial biofertilizer dates back to 1866 which was first patented in 1896 (Woronin 1866; Nobbe and Hiltner 1896). Since the discovery of the growth promoting effects of *Azotobacter* (for a review see Behl et al. 2007) and *Azospirillum* (for a review see Okon and Labandera-Gonzalez 1994) the systematic research on such rhizobacteria is growing. New species and strains are constantly being discovered and tested and also their economic importance is increasing. But not only bacteria are being studied, also fungi (Goos et al. 1994; Gan et al. 2005; Singh and Reddy 2012), even yeasts (Javaid and Mahmood 2010) and of course AMF, the main topic of this thesis. Table 1 shows the main groups of biofertilizers and their main functional traits if known.

Table 1: Groups of microorganisms identified as biofertilizers.

Functional group (if known)	Biofertilizer group	Examples of species
P mobilizers	Arbuscular mycorrhizal fungi (AMF)	*Claroideoglomus etunicatum, Gigaspora rosea, Glomus caledonium, G. clarum, G. hoi, G. leptotichum, G. mosseae, Entrophosphora colombiana, Rhizophagus fasciculatum, R. irregularis*
P mobilizer Nutrient cycling Pathogen resistance	Other Fungi (F)	*Actinomycetes, Aspergillus niger, A. tubingensis, Penicillium bilaii, P. brevicompactum, P. solitum, Piriformopora indica, Trichoderma atroviride, Trichoderma harzianum*
N fixers P solubilizers Plant hormone producers	Plant Growth Promoting Rhizobacteria (PGPR)	strains of *Bacillus megaterium, B. polymixa, Enterobacter* sp.

Functional group (if known)	Biofertilizer group	Examples of species
Plant hormone producers	Plant Growth Promoting Rhizobacteria (PGPR)	*Bacillus circulans, B. mycoides, B. pummilus, B. simplex, B. subtilis, Burkholderia tropica, Citrobacter freundii, Kurthia* sp., *Ochrobactrum anthropic, O. ciceri, Rhodobacter capsulatus, Rhodopseudomonas* sp., *Rhodotorula glutinis, Variovorax paradoxus*
S solubilizers	Sulphate solubilizing bacteria (SSB)	*Thiobacillus sp., T. thioxidans*
P solubilizers Plant hormone producers	Phosphate solubilizing Bacteria (PSB)	*Arthrobacter chlorophenolicus, Bacillus firmus, B. megaterium, B. mucilaginous, Burkholderia caryophylli, Enterobacter asburiae, Microbacterium arborescens, Paenibacillus* sp., *P. polymixa, Penicillium bilaii, Providencia* sp., *Pseudomonas aeruginosa, P. argentinensis, P. cepacia, P. chlororaphis* subsp. *aurantiaca, P. diminuta, P. fluorescens, P. fragi, P. jesseni, P. marginalis, P. paleroniana, P. putida, P. striata, P. syringae, P. tolasii, Serratia marcescens, Staphylococcus saprophyticus*
N fixers Plant hormone producers	Free living diazotrophs	*Azotobacter chrooccocum, Azotobacter brasilense, Beijerinckia indica, Klebsiella pneumoniae, strains of Bacillus megaterium, Anabaena cylindrica, Anabaena variabilis, Aulosira fertilissima, Nostoc muscorum and Tolypothrix tenuis, Gloeotrichia, Nostoc, Calothrix, Aphanothece spp., Anabaena oscillaroides, Brevundimonas diminuta,*
N fixers Plant hormone producers	Symbiotic diazotrophs	*Azospirillum brasilense, Azospirillum lipoferum*
N fixers Plant hormone producers	Rhizobia	*Mesorhizobium ciceri, Rhizobium leguminosarum, Bradyrhizobium japonicum, Bradyrhizobium diazoefficiens*

Several reviews have been published besides original articles. Okon and Labandera-Gonzalez (1994) reviewed inoculation of *Azospirillum* in several crops, as Veresoglou and Menexes (2010) did again for wheat only. Pereg and McMillan (2015) reviewed the specificity of *Azospirillum* strains. Behl et al. (2007) reviewed interactions of AMF and *Azotobacter* on wheat. McGonigle (1988) and Berruti et al. (2016) analyzed the potential of AMF as

biofertilizers. Lehmann et al. (2014) studied the role of AMF for the uptake of Zn in crop plants, and Lehmann and Rillig (2015) studied the uptake of copper, manganese and iron; both studies analyzed field and greenhouse studies. Rubin et al. (Rubin et al. 2017) studied the influence of PGPR especially under drought conditions. Hence considerable knowledge exists on the effects of single categories of biofertilizers on specific target crops.

Two countries show an increased interest in the study and application of biofertilizers and most published field studies originate there. Both India and Iran have government programs to promote biofertilizers. India is largely dependent on agriculture with poor soils in many parts and a fast population growth and Iran has semi-dry to dry climate with poor soils as well, which may explain their engagement in biofertilizers. India has a long history in the research and application of biofertilizers, which are produced by agro industry corporations, state agriculture departments, national biofertilizer development centers, state agriculture universities and private sector (Singh et al. 2014). The government of India supports laboratories producing biofertilizers and also subsidizes the purchase of biofertilizers (Press Information Bureau; Government of India; Ministry of Agriculture 2014). The ministry of Agriculture of India reports for the year 2014 an annual production of carrier based biofertilizers of about 80.700 tons and 4000 L of liquid based biofertilizers (Ministry of Agriculture 2016). In Iran the production and application of biofertilizers have been encouraged since the 2000s. Three main private companies produce biofertilizers. The main types of biofertilizers include: *Thiobacillus* (Sulphur-oxidizing bacteria) along with zinc, granulated phosphate solubilizing bacteria and N fixing *Azotobacter* (FAO- Land and Plant Nutrition Management Service 2005). An overview for other countries has been provided by IPNI (2011).

Symbiotic arbuscular mycorrhizal fungi and their use as biofertilizers

The mutualistic symbiosis between AMF and plants is ancient and dates back to the time, 460 million years ago, when plants started to live on land. It is thought that, the inability of aqueous plants to form an extensive branched root system brought in the AMF with their filamentous hyphae (Smith et al. 2008a). They provide plants with water, but also nutrients like P, N, S and micronutrients like Zn. To exchange these nutrients with the products of plant's own photosynthesis, they develop arbuscules inside the cells of the root, tiny treelike structures with a large surface (Smith et al. 2008b). AMF have lost the ability to survive alone and are considered obligate biotrophs. Because this symbiosis existed before plants species have diversified into thousands of species, the diversity of AMF remained low. Most plant species

kept living in this symbiosis thus there was no selection pressure on the AMF and the level of specificity of the interaction stayed low (Sanders 2003).

The external mycelium can cover large areas and connect multiple plants of the same species but also other species. The so called common mycorrhizal network (CMN) enables an exchange of soluble nutrients but also carbon molecules produced by the plant (Lerat et al. 2002; Simard and Durall 2004). One plant can be colonized by up to 20 different AMF species (Parniske 2008). The exchange is influenced by the competition of the AMF species but also the plant species and some interactions are beneficial to both partners while others are termed parasitic when one partners receives more than it gives (Smith and Read 2008). These interactions shape the plants performance but even the diversity of the plant community is shaped by the belowground community composition (van der Heijden et al. 1998).

When AMF are used as biofertilizers they are multiplied either in pot culture of which soil and cut roots are applied to the seed furrows or the nursery or they are multiplied on petri dishes on root organ cultures where the spores can be harvested and used to coat the seeds prior to sowing. Their yield promotion has been studied in a meta-analysis, similar to the one in this thesis, and can be substantial (Lekberg and Koide 2005).

The potential of intercropping or mixed cropping

Intercropping or mixed cropping describes a farming system with two or more crop species, or genotypes, growing together and coexisting for a certain time. It is an ancient farming practice and is still practised by many subsistence farmers. Such practices have not survived the introduction of the modern highly mechanized intensive agriculture. However now mixed cropping or intercropping is discussed again as an option for a sustainable intensification of agriculture (Brooker et al. 2015). It increases the diversity in agriculture by combining two or more crop species or genotypes. They are planted at the same time or they overlap for a certain time also called relay cropping. Plants can be sown by hand or in regular rows. This practice can increase yields on a given piece of land compared to monocropping of one of the crops and this is called overyielding.

Interspecific plant interactions change the use of resources by making complementary use of them or by facilitating the uptake for the other crop (Vandermeer 1989; Zhang and Li 2003). A complementary use of resources can happen through various mechanisms like complementary root architecture which enables the plants to make the best use of space for taking up nutrients and water. Facilitation can be achieved by combining cereals with legumes which can fix nitrogen and may make a part of this nitrogen available to cereals (Hauggaard-Nielsen and

Jensen 2005; Li et al. 2007). But also pest and pathogen pressure is reduced in intercropping systems: Mixing genotypes of a given crop may combine different modes of resistance to pathogens and reduces yield losses (Mundt 2002). But the coexistence of the two crops also depends on abiotic factors like nutrient availability, soil type or climate (Wang et al. 2007). Mixed cropping can act as a buffer against extreme events when one crop is more resilient than the other which stabilizes yields over time. Because of the increased resilience it is often discussed to be especially suited for marginal lands (Qiao et al. 2015).

Mixed cropping has been shown favourable for the P nutrition. Studies have found that P solubilizing plants like faba bean or peanuts were able to also facilitate the access to P for other plants (Li et al. 2003, 2010; Xia et al. 2013). Here complementarity describes the use of different P pools, chemical or from different soil depth, by the different plants of the system. Facilitation describes the facilitated uptake of phosphorus by the other crop through for example the excretion of root exudates. Exuded organic acids solubilize inorganic P, while root-borne phosphatases hydrolyse organic P (Hinsinger 2001; Vance et al. 2003; Hinsinger et al. 2011). The P recovery of phosphate fertilizers is usually rather low (Syers et al. 2008). Soils especially in the tropics are low in soil available phosphorus and high in metals like iron, aluminium and calcium which immobilize phosphorus. It is thus of paramount importance to study agricultural systems which can make use of immobile forms of P and improve the use efficiency of phosphate fertilizers.

Pigeon pea (*Cajanus cajan*)-finger millet (*Eleusine coracana*) intercropping is a common intercrop system in southern India. Pigeon pea is generally a popular crop for mixed cropping in India with 65 mixtures recorded (Ahlawat et al. 2005). Pigeon pea is a deep rooting legume with rhizobial nitrogen fixation. Finger millet on the other hand is a shallow rooted cereal with C4 carbon fixation. Both crops have low requirements in nutrients and are cultivated on marginal lands. The combination of the two has been shown to be superior to monocropping (Mathimaran et al., in preparation). Through the excretion of piscidic acid pigeon pea has a strong ability to solubilize immobile forms of P like calcium phosphate, aluminium phosphate or iron phosphate (Ae et al. 1990) which are the natural products of P immobilisation in highly eroded soils and could thus facilitate other plants. There is also a temporal complementarity between the two as finger millet is harvested earlier, hence the peak of nutrient uptake is shifted. If pigeon pea is grown in a nursery before the onset of the monsoon and the regular planting time, they have the advantage to be larger at time of flowering thereby having higher yields (Praharaj et al. 2015). The success though depends also on the onset of the monsoon and planting time (Pavan et al. 2011).

The soil microbial community: hidden neighbors of the plants

The indigenous soil microbial community is an important factor for the success of inoculating biofertilizers. Although they are inoculated very close to the seed and do not need to look for a habitat themselves, for the successful inoculation they need to be able to compete with the indigenous microfauna.

The soil microbial community has long been a black box because only few microorganisms could be cultivated and studied in the lab. With the introduction of next generation sequencing the big data era has also reached the community analysis of soil microbes which allows to investigate their complex relationship in high detail. However, the interactions of various microorganisms which form complicated networks are hard to predict (Fuhrman 2009; Zhou et al. 2010).

Microbial communities are under the influence of many factors. Depending on the soil composition, location and physico-chemical factors the microbial communities differ (Tecon and Or 2017). One important factor for the soil microbial community in agricultural soils is the management of the soils by tillage, fertilization or crop rotation. To understand the factors which make biofertilizers inoculations work, the influence of the soil management needs to be understood better. A rich microflora is expected to buffer the changes by an introduced species better and inoculants were shown to be more effective when the microbial biomass in the soil was low. (Fließbach et al. 2009). Bacterial community changes are also caused by seemingly unimportant factors like plant age (Lerner et al. 2006; Piromyou et al. 2013) which can be stronger than the inoculation effect itself. Yet due to the sensitivity of microbial communities in each site and even year the inoculants will meet a different community of microorganisms (Tecon and Or 2017).

Functional diversity describes the diversity of enzymes for the degradation of organic matter, the production of antibiotics or genes critical biogeochemical cycles. It is next to biodiversity a concept to gain better understanding of the soil processes involving microbes. After inoculation a community is expected to have an increased functional diversity. Kohler et al. (2010) found a change in the fungal diversity by the inoculation of lettuce plants with PGPR and/or AMF and this increased fungal diversity increased the diversity for the utilization of carbon sources. Also Gupta et al. (2016) found an increase in functional diversity. After inoculating pigeon pea with a consortium of *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma harzianum* they found an increase in the nitrogen fixing group of *Azospirillum* and the known PGPR agent *Bacillus simplex*. However community changes cannot always be assigned to positive or

negative changes in the functional diversity (Ramakrishnan et al. 2017); possible conclusion depend also on the methods used.

It is unknown how these changes influence ecosystem services over the season. There is also the potential that an inoculant becomes invasive in a soil environment thus changing ecosystem structure, functioning and services. Using native microorganisms as inoculants will reduce the risk of the endeavor, but then diversity of microorganism is found on such a microscale (Torsvik and Øvreås 2002) that the definition of native actually becomes a problem.

High-throughput sequencing technology will help to improve the mechanistic understanding between microbial diversity and ecosystem functioning (Fitter et al. 2005; Zhou et al. 2015). With the already existing and continuously increasing databases it is possible not only to sequence these uncultivated microorganisms but even to identify the biochemical pathways of not only one species but of the whole community via already studied pathways from the lab (Knief 2014). It is however unresolved by DNA sequencing whether the extracted DNA belongs to an active microbe or not. Estimates suggest that only a few % of the total microbial biomass found in a soil are active while most exist in dormant or inactive forms (Blagodatskaya and Kuzyakov 2013). Meta-transcriptomic or meta-proteomic studies will be able to resolve this issue but also drive community analysis to another level of complexity. A very similar topic is the study of suppressive soils where crop plants suffer less from certain soil pathogens and key responsible bacterial taxa and also genes could be identified with the study of the microbiome (Mendes et al. 2011).

Aims of the thesis

I. The inoculation of biofertilizers in arable crops has been proven many times to efficiently improve plant growth besides many other beneficial effects. Yet the difficulties in predicting the success of microbial inoculants hamper their spread in agriculture. Based on the numerous studies published since the 1980s it is hypothesized that it is possible to extract key factors for the success of biofertilizers from these studies.

Secondly, the aim is to study the possibility to use AMF and PGPR as “biofertilizers” in an intercropping systems in Indian agriculture, planting pigeon pea (*Cajanus cajan*) seedlings pre-inoculated with AMF into a field sown with finger millet (*Eleusine coracana*). This system has also been studied in field trials in India in the “Biofertilization and Bioirrigation network” (BIOFI) within the Indo-Swiss Collaboration in Biotechnology (ISCB). With the combination of these three elements, including plant diversity and soil organisms, we want

to make optimal use of their ecological traits and utilize the functional diversity to establish a more sustainable agricultural system with better use of resources.

II. When the inoculated pigeon pea seedlings are planted out into the field the hyphae will start to spread in the soil and may also reach the roots of the finger millet. To understand the system, it is necessary to study the growth of the hyphae and find out whether the roots of the finger millet are colonized, at what time and how it influences the growth performance of finger millet.

III. The ability of pigeon pea to solubilize immobile sources of phosphate raises the question whether this will be of benefit to neighbouring finger millet plants. AMF are well known for their role in the P uptake of plants. Of their role in the uptake of immobile forms of P much less in known. Also their ability to transport allelochemicals (Achatz and Rillig 2014) raises the question whether root exudates are transported, facilitating the uptake of immobile P for one or both crops.

IV. The soil microbial community plays an important role for the success of inoculants. The question is how the microbial community is affected by the inoculants and vice versa their survival if affected by the indigenous community. For that it is important to be able to trace the inoculum in the soil during the cropping season. But also other factors like whether inter- or moncropping affects the soil microbial community are little studied.

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Improving Crop Yield and Nutrient Use Efficiency via Biofertilization—A Global Meta-analysis

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The application of microbial inoculants (biofertilizers) is a promising technology for future sustainable farming systems in view of rapidly decreasing phosphorus stocks and the need to more efficiently use available nitrogen (N). Various microbial taxa are currently used as biofertilizers, based on their capacity to access nutrients from fertilizers and soil stocks, to fix atmospheric nitrogen, to improve water uptake or to act as biocontrol agents. Despite the existence of a considerable knowledge on effects of specific taxa of biofertilizers, a comprehensive quantitative assessment of the performance of biofertilizers with different traits such as phosphorus solubilization and N fixation applied to various crops at a global scale is missing. We conducted a meta-analysis to quantify benefits of biofertilizers in terms of yield increase, nitrogen and phosphorus use efficiency, based on 171 peer reviewed publications that met eligibility criteria. Major findings are: (i) the superiority of biofertilizer performance in dry climates over other climatic regions (yield response: dry climate +20.0 ± 1.7%, tropical climate +14.9 ± 1.2%, oceanic climate +10.0 ± 3.7%, continental climate +8.5 ± 2.4%); (ii) meta-regression analyses revealed that yield response due to biofertilizer application was generally small at low soil P levels; efficacy increased along higher soil P levels in the order arbuscular mycorrhizal fungi (AMF), P solubilizers, and N fixers; (iii) meta-regressions showed that the success of inoculation with AMF was greater at low organic matter content and at neutral pH. Our comprehensive analysis provides a basis and guidance for proper choice and application of biofertilizers.

Keywords: meta-analysis, biofertilizer, microbial inoculants, agricultural productivity, nitrogen use efficiency, phosphorus use efficiency, arbuscular mycorrhizal fungi, PGPR

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INTRODUCTION

The current alarming rate of decline of earth's natural resources, particularly of the reserves of rock phosphate and fossil fuel, is of great concern for the future of agriculture, particularly in developing countries (St.Clair and Lynch, 2010). Not surprisingly, sustainable crop production remains a major global challenge and has drawn increasing attention among policy makers, business, and the scientific community (Seufert et al., 2012; Wezel et al., 2014). Efforts to mitigate the declining mineral nutrient reserves are currently major topics of research but the perturbance of the global biogeochemical cycles, mainly driven by the use of mineral fertilizers, remains a serious problem (Kahiluoto et al., 2014).

Microbial inoculants, so-called biofertilizers, are a promising technology to reduce the use of conventional inorganic fertilizers. Many of them can serve as biofertilizers as they are able to fix nitrogen (N), help to access nutrients such as phosphorus (P) and N from organic fertilizers and soil stocks, improve drought tolerance, improve plant health or increase salt tolerance (Vessey, 2003; Arora, 2013). The effects of biofertilizer applications have often been inconsistent, hindering their widespread adoption by farmers. The reasons can be manifold, such as soil conditions, strain identity, or host genotype. Yet, the long history of research offers a great reservoir to identify key influencing factors. Numerous reviews on microbial inoculants have been published, but quantitative results are scarce. For example, McGonigle (1988), Lekberg and Koide (2005), and Berruti et al. (2016) analyzed the potential of AMF (arbuscular mycorrhizal fungi) as biofertilizers. Rubin et al. (2017) studied the influence of PGPR (plant growth-promoting rhizobacteria) especially under drought conditions. Nevertheless, what is missing is a comprehensive quantitative analysis over all biofertilizers and across all target crops and climatic conditions at global scale.

Here, we conducted a quantitative evaluation of the pertinent literature in the form of a meta-analysis. Its objective was to quantify the effect of biofertilizers on the performance indicators crop yield and P and N nutrient use efficiencies.

The following hypotheses were addressed: (i) across all studies, biofertilizer show a significant positive effect on crop yield and nutrient use efficiency; (ii) there is a difference in biofertilizer response between categories of crops; (iii) climate is a major factor for the constituency of soil biodiversity, soil fertility and soil carbon content, and thus the performance of biofertilizers; (iv) P availability is a limiting factor in many soils. P levels are expected to influence activity and thus effectivity of biofertilizers. Especially phosphate-solubilizing bacteria and AMF are expected to be affected by P levels.

MATERIALS AND METHODS

Search Strategy

Peer reviewed publications (and the reference lists from these publications) were searched for between May 2015 and February 2016 in Web of Science by Thomson Reuter, Scopus by Elsevier and Google Scholar with the following keywords "biofertilizer OR biofertiliser OR microbial inoculants." Only studies using data from field trials to more closely reflect real farming practices and providing separate data for each treatment and written in English language were selected. Studies were only included when they had conducted pairwise comparison between the application of a biofertilizer to a non-treated control under the same pedo-climatic conditions (e.g., temperature, precipitation, soil texture, and type), and if the biofertilizers had been tested under the same input level of inorganic and organic fertilizers as the paired non-inoculated control. Studies had to report the treatment mean of yields, its standard deviation (SD) and number of replications (n) to calculate the different use efficiencies and effect sizes. When fertilizer was applied the amount and type of fertilizer was required to calculate nutrient-use efficiencies for phosphorus (P) and nitrogen (N). Field trials were not included when soils were previously fumigated or heat sterilized to obtain a control without soil biota, because nutrients may be released, soil microbial community disturbed and inoculation success put at risk (Smith and Read, 2008a). If data were missing or only supplied in summarized format, authors were contacted to obtain these data. A total of 633 possible studies were identified, 222 were excluded after a first screening for greenhouse studies (except three studies with tomato grown under commercial conditions) and reviews and again 240 because they did not match eligibility criteria mentioned above (see flow diagram in **Figure S1**).

Data Sources

One hundred and seventy-one studies (see study list in **Supplementary Data Sheet S1**) proved to be eligible for our meta-analysis enabling us to generate 1,726 pairwise comparisons.

Data Preparation and Descriptive Statistics

All data was extracted and compiled in an excel file. If the data were only available in graph format, Plot Digitizer Version 2.6.6 (http://plotdigitizer.sourceforge.net) was used. The data was structured after biofertilizers, crops and climate. **Tables 1** and **4** summarize the characteristics of crop and climate categories for the number of included studies, amount of fertilizer applied and climate representation. pH was usually given as measured in water. If pH was measured in $CaCl_2$, conversion was calculated (Land Resources Management Unit, Institute for Environment and Sustainability, 2010). If the method was missing it was assumed to be measured in water. Soil pH was later used as a control variable for meta-regression.

Bulk density was only available for 10 studies. For the others bulk density was estimated with the pedo-transfer function (Post and Kwon, 2000). Bulk density was necessary to convert soil available P from mg/kg to kg/ha. Soil available phosphorus was calculated to a depth of 30 cm. Soil available phosphorus was measured mostly with the method by Olsen, but also with Bray, Mehlich, and AB DTPA. Yet in many cases the method was not given. Yli-halla (2016) state that usually there is a rough agreement between the results obtained with different extraction methods in non-calcareous soils, but in calcareous soils the results of acidic and basic extractants usually have a poor correlation. Hence the values of soil available phosphorus cannot be seen as absolute values but only as an indicator for the real values. Soil available phosphorus was calculated to provide another perspective on phosphorus other than P use efficiency (PUE). Since no formula exists to account for available phosphorus from soil and fertilizer we conducted a meta-regression with the sum of soil available P and fertilizer P. Thus, for a comprehensive picture, we provide three different analyses of functional biofertilizer categories to P.

Meta-analysis

A random-effects model was chosen as the statistical model for the meta-analysis (Viechtbauer, 2010b). In a meta-analysis, ideally, independent estimates should be aggregated (Borenstein et al., 2009), but in reality, and also in this meta-analysis this cannot be fully assured. Independence is violated in the cases,

TABLE 1 | Database as related to different crop categories, climatic zones and nutrient inputs.

	Cereals	Root crops	Legumes	Vegetables	Other crops
Number of studies*	86	8	38	17	28
Number of pairwise comparisons**	681	137	521	142	184
Coverage of climatic zones (after Koeppen)	Aw, BSh, BSk, BWh, Cwa, Cfa, Csa, Cfb, Cwb, Dsb, Dsa, Dfb, Dwb	Aw, Cfb, Cwa, Csa, Dfb	Aw, BSk, BSh, BWh, Cwa, Cwc, Csa, Cwb, Dwa, Dsb, Dsa,	Aw, BWh, Csa, Cwa, Cfa, Cwb, Dfb,	Am, Aw, BSk, BWh, BSh, Cwa, Cwb, Csa Dsb,
Coverage of continents	5	3	4	4	3
Average N applied (kg ha^{-1}) (mean/median ± SD)	100.7/80.0 ± 84.6	127.2/102.5 ± 75.4	44.2/22.5 ± 56.6	159.2/200.0 ± 63.0	158.2/110.0 ± 214.7
Average P applied (kg ha^{-1}) (mean/median ± SD)	50.3/40.0 ± 37.2	56.3/52.4 ± 35.6	33.0/25.0 ± 24.8	53.1/53.7 ± 19.1	56.4/40.0 ± 56.2
Percent unfertilized of pairwise comparisons	27.90	0	19.77	9.86	8.15

*Averages for fertilizer applications were only calculated if fertilizer were applied; *Some studies appear in more than one crop category resulting in a higher (177) sum of studies than reported (171); **Legumes comparison with rhizobia as control (61 comparisons from 12 studies) are not included in the category for legumes resulting in lower (1665) sum of comparison than the reported (1726).*

where several treatments are compared to the same control. It is likely also violated for the cases where study results over several years from the same comparison plots were not averaged but included separately in the meta-analysis. In both cases, we retained all data because the aim of the meta-analysis was to include as much information as possible. For the second case, N use efficiency (NUE) and P use efficiency (PUE) likely depend strongly on the annually different climate conditions, thus rather mitigating dependence. If values were supplied as an average over years, replicate numbers of each year were multiplied by the number of years. The random-effects model assumes that the single effect size depends on the study context and that studies differ in their methods and sample characteristics. As a result, there are different effect sizes among all studies. Since the true effect size and its variance are not known the restricted maximum-likelihood estimator (REML) was used (Viechtbauer, 2010b). Outliers were identified via DFBETAS values inside the R package "metafor" (Viechtbauer, 2010a).

Effect Sizes and Their Modeling

Effect sizes indicate the magnitude of the effect of the improved practice over the control practice concerning yield responses and nutrient use efficiency (Borenstein et al., 2009). In this study, the percent increase in dry matter yields was used for comparing yields and raw mean difference was used as effect size measure for PUE and NUE, calculated as the log transformed ratio of the mean.

Performance Indicators

In this study, we evaluated quantitatively the effects of all categories of biofertilizers on crop yield, PUE and NUE, with a main focus on relative crop yield. Key characteristics of the studies can be found in the **Supplementary Data Sheet S2**. Yield is defined as harvested dry main product, in form of grains, fruits, tubers or shoots. Dry weight had to be calculated for most studies. If the water content was not available, values were taken from Church and Bowes (Church and Bowes, 1966). PUE was calculated as the yield increase of dry main product per unit of P fertilizer input, and NUE accordingly as the yield increase per unit N fertilizer input referring to the agronomic efficiency of P and N, respectively (Ladha et al., 2005).

The following formulae were used:

$$Yield\ response\ (\%) = \frac{Yield_{\ inoculated} \times 100}{Yield_{non-inoculated}} \quad (1)$$

$$\Delta PUE = \frac{Yield\ (kg\ ha^{-1})}{Fertilizer\ P(kg\ ha^{-1})_{\ inoculated}} - \frac{Yield\ \left(kg\ ha^{-1}\right)}{Fertilizer\ P\left(kg\ ha^{-1}\right)_{\ non-inoculated}} \quad (2)$$

$$\Delta NUE = \frac{Yield\ (kg\ ha^{-1})}{Fertilizer\ N(kg\ ha^{-1})_{\ inoculated}} - \frac{Yield\ (kg\ ha^{-1})}{Fertilizer\ N(kg\ ha^{-1})_{\ non-inoculated}} \quad (3)$$

Given the lack of data for estimating or modeling these additional N sources and P, the chosen approach to calculate PUE and NUE is most adequate. Nevertheless, it may lead to different effects regarding soils and nutrient loss to the environment. In case higher PUE or NUE are observed with biofertilizers with identical P and N fertilizer inputs, the biofertilizer must have resulted either in more efficient uptake of those inputs, or in making additional inputs from the soil pool available. In the first case, nutrient mining effects of soils is unlikely and potential runoff is reduced; in the second case, some nutrient mining may occur, if runoff is not reduced, e.g., if nutrients mobilized from the soil and taken up by the plant are replaced in the soil by nutrients from the fertilizer input. With the available data, we cannot discern these two cases. We report yield response in percent thereby neglecting the actual values and their size. Percentage values are necessary to normalize the yields. But percentage values are insensitive

to whether the yields are already at a maximum or whether there are yield gaps in terms of other management techniques which pose a different potential to decrease or increase yields by the inoculated biofertilizers. The calculation follows the general methods used by Batten (1992). Due to lack of information on the soil types of the studies, which are crucial for the absorption of phosphorus, we believe that this method reflects PUE the best. NUE was calculated as yield of dry product by N fertilizer input. This calculation is widely used for studies in an agricultural context and referred to as agronomic nitrogen use efficiency (Yadav, 2003; Ahmad et al., 2009; Zhang et al., 2016). Yet it is criticized because it does not reflect N inputs from atmospheric deposition, nitrogen fixation and mineralization from organically bound nitrogen (Godinot et al., 2014). These inputs were not reported and are difficult to model. Our calculation is thus an apparent nitrogen use efficiency and needs to be looked at as an indicator for total nitrogen use efficiency.

Crop and Biofertilizer Categories

Data were grouped into the main crop categories cereals, root crops, legumes, and vegetables. Spices like fennel or anise, cotton and oil crops were classified as other crops (see **Table 2**). To structure the effects of the microbial inoculants, they were classified for their P solubilization and N fixation activity. In this way, it was also possible to account for combined inoculation with different inoculants. The information on the main traits of the inoculants was taken from the studies and further literature sources. Thus, five categories were distinguished: Arbuscular mycorrhizal fungi, P solubilizers, N fixers, a combination of both P solubilization and N fixation, either in one strain or by applying two strains, and other biofertilizers with unspecified modes of action, also in combination with AMF (see **Table 3**). It allowed to classify biofertilizers according to their needs of phosphorus by relating their effect to plant available P in soil, thus providing direct guidance to practitioners and farmers at which level which biofertilizer is most promising.

Climate Classification and Other Site Characteristics

The study locations were classified according to an updated Köppen climate classification (Peel et al., 2007). Thereby the studies were split into dry (BSh, BSk, BWh, Csa) and tropical climate (Aw, Am, Cwa, Cwb, Cwc, Cfa,), continental climate (Dfb, Dsa, Dwa, Dwb, Dsb), and oceanic climate (Cfb). In many studies, the experiments were performed under irrigated conditions or planted in the rainy season. Thus the climate classification is often rather an indicator for potential soil fertility and related indicators such as soil carbon than climate itself (**Table 4**). Because regions with Mediterranean climate have low soil carbon contents they were grouped into dry climate as well. This grouping enabled us to make a cross comparison of different biofertilizer categories and to identify key conditions for the successful application of biofertilizers.

Data Analysis

The dataset used for this study is available in the **Supplementary Data Sheet S2**. The meta-analysis was conducted with R Software Version 3.2.3 and the interface R-Studio Version 0.99.491 using the "metafor" package (Viechtbauer, 2010b). Also the meta-regressions were calculated

TABLE 2 | Crops included in this meta-analysis.

Crop category	Crops included
Cereals	Barley, durum wheat, rice, spring wheat, winter wheat, pearl millet, maize, sorghum, kamut, silage maize, ryegrass, finger millet
Legumes	Blackgram, chickpea, peanut, horsegram, kidney bean, mung bean, fenugreek, lentil, snap bean, soybean, runner bean, pigeon pea
Root crops	Garlic, potato, turmeric, sugar beet, cassava
Vegetables	Eggplant, tomato, cabbage, watermelon, pepper, okra, cucumber, melon
Other crops	Dill, anise, rapeseed, cotton, sesame, fennel, coriander, sunflower, mustard, sugarcane

TABLE 3 | Categorization of microbial inoculants according to species characteristics and functionality.

Category	Species
AMF	*Entrophosphora colombiana*, *Glomus caledonium*, *G. clarum*, *G. etunicatum*, *G. fasciculatum*, *G. hoi*, *G. intraradices* (new name: *Rhizophagus irregularis*), *G. mosseae*, *Gigaspora rosea*
P solubilizers	*Arthrobacter chlorophenolicus, Bacillus firmus, B. megaterium, B. mucilaginous, Burkholderia caryophylli, Enterobacter asburiae, Microbacterium arborescens, Paenibacillus* sp., *P. polymixa, Penicillium bilaii, Providencia* sp., *Pseudomonas aeruginosa, P. argentinensis, P. cepacia, P. chlororaphis* subsp. *aurantiaca, P. diminuta, P. fluorescens, P. fragi, P. jesseni, P. marginalis, P. paleroniana, P. putida, P. striata, P. syringae, P. tolasii, Serratia marcescens, Staphylococcus saprophyticus*
N fixers	*Anabaena azollae, A. cylindrica, A. oscillaroides, A. variabilis, A. torulosa, Aphanothece* spp., *Aulosira fertilissima, Azolla caroliniana, Azospirillum brasilense, A. lipoferum, Azotobacter brasilense, A. chrooccocum, Bacillus polymyxa, B. subtilis, Beijerinckia indica, Bradyrhizobium diazoefficiens, B. japonicum, Brevundimonas diminuta, Burkholderia vietnamensis, Calothrix* sp., *C. elenkinii, Gloeotrichia* sp., *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Klebsiella pneumoniae, Mesorhizobium ciceri, Nostoc muscorum, N.* sp., *Rhizobium leguminosarum, Staphylococcus* sp., *Tolypothrix tenuis*
N fixers plus P solubilizers	Strains of *Bacillus megaterium, B. polymixa, Enterobacter* sp., joint inoculations of P solubilizers and N fixers
Other biofertilizers	*Actinomycetes, Aspergillus niger, A. tubingensis, Bacillus circulans, B. mycoides, B. pummilus, B. simplex, B. subtilis, Burkholderia tropica, Citrobacter freundii, Kurthia* sp., *Ochrobactrum anthropic, O. ciceri, Penicillium brevicompactum, P. solitum, Piriformopora indica, Rhodobacter capsulatus, Rhodopseudomonas* sp., *Rhodotorula glutinis, Thiobacillus* sp., *T. thioxidans, Trichoderma atroviride, T. harzianum, Variovorax paradoxus,* joint inoculations with AMF

TABLE 4 | Database as related to climatic zones and nutrient inputs.

	Tropical climate	Dry climate	Continental climate	Oceanic climate
Nr of studies[a]	70	71	17	8
Nr of pairwise comparisons	686	718	152	110
Coverage of continents	5	5	3	3
Average N applied (kg ha^{-1}) (mean/median ± SD)	90.8/60 ± 88.2	120.5/90 ± 132.1	78.2/80 ± 58.3	65.3/47.5 ± 45.4
Average P applied (kg ha^{-1}) (mean/median ± SD)	47.3/38 ± 35.1	48.6/35.7 ± 40.7	37.8/34.9± 29.4	55.0/70.0 ± 30.2
Average OM% (mean/median ± SD)	1.69/0.88 ± 1.59	1.02/0.95 ± 0.79	2.37/1.8 ± 1.85	4.82/4.18 ± 2.85
Average pH (mean/median ± SD)	6.66/6.80 ± 1.20	7.81/7.80 ±0.34	7.16/7.15 ± 0.61	5.55/5.50 ± 0.98

Averages for fertilizer applications were only calculated if fertilizers were applied.
[a]*Five of the studies analyzed were excluded because they could not be assigned unequivocally to one climate zone.*

within this package by designating moderator variables which were used to calculate a mixed effects model (**Figures 6–8**). Selection bias was assessed with funnel plots (**Figure S2**) and outlier analysis was undertaken via DFBETAS values inside the R package "metafor" (Viechtbauer, 2010b).

Missing Values

Sometimes the nutrient content of organic fertilizers was not available, values were then taken from a booklet within a national project on organic farming by the Indian government (Chandra, 2005). Where bulk density was missing, it was estimated with the pedo-transfer function by Post and Kwon (2000):

$$\mathrm{BD} = \frac{100}{\left(\frac{OM_{conc}}{0.244}\right) + \left(\frac{100 - OM_{conc}}{1.64}\right)} \quad (4)$$

where 0.244 is the bulk density of organic matter, 1.64 the bulk density of soil mineral matter, and OM_{conc} the concentration of soil organic matter (%), which was estimated according to Nelson and Sommer (1982), if necessary:

$$OM_{conc} = 1.72 \times SOC_{conc} \quad (5)$$

Missing errors were estimated from the average reported standard deviations in percent, differentiated per crop groups. For cereals, the standard deviation (SD) was 15.2%, for legumes SD 5.5%, for melon and water melon SD 35.9%, for vegetables SD 11.2%. For maize (SD 10.6%), cotton (SD 14.0%), rice (SD 14.18%), mustard and rapeseed (SD 10.2%) values were averaged within each type of crop. Average of all were applied for anise, fennel, dill, sesame, sunflower, coriander, garlic, ryegrass, turmeric, silage maize, potato, sugarcane SD 12.0%. The standard deviation in yield as a percentage was used to estimate the error in PUE and NUE.

Bias Assessment

It cannot be excluded that there was a certain publication bias within the results. In order to find out whether there was a publication bias in the meta-analysis "funnel plots" were used to detect a possible publication bias. The trim and fill method was used to help interpretation as proposed by Duval and Tweedie (2000a,b) and Duval (2005). Modest bias was found in some groupings (**Figure S2**), but no studies were excluded.

RESULTS

Our comprehensive meta-analysis with studies from all over the world (**Figure 1**) revealed that biofertilizers were found to be most effective in dry climates (**Figure 2**). Biofertilizer also improved PUE and NUE greatly. Furthermore, we found that biofertilizers possessing both N fixing and P solubilizing traits have the highest potential to improve the crop yields (**Figure 3**). Interestingly, AMFs, known for facilitating P nutrient uptake in plants, were on par with applications of biofertilizers with the combined traits of N fixation and P solubilization, indicating the big potential of AMFs as sole biofertilizer for most crops and climatic situations.

Yield Impact of Biofertilizers by Climate

Averaged across all biofertilizer categories, yield was increased the most in dry climates (+20.0 ± 1.7%), followed by tropical climates (+14.9 ± 1.2%), oceanic climates (+10.0 ± 3.7%), and continental climates (+8.5 ± 2.4%) (**Figure 2**). For interpretation, it is important to keep in mind that 45% of the comparisons in dry climate were conducted in the presence of irrigation. In a separate analysis of the data from dry climates, we found a significant difference in the yield increase under irrigated conditions with +15.9 ± 2.0% (316 comparisons, 39 studies) and under rainfed conditions with +21.0 ± 3.1% (274 comparisons, 20 studies). In dry climates soils had the highest pH and the lowest soil organic matter (OM) content; here, the highest amount of N fertilization was used (**Table 4**). However, in all climates, the variation of fertilizer application levels within the trials was high.

Yield Impact of Different Biofertilizer Categories

AMF, other biofertilizers and the application of biofertilizers with both functional traits—N fixation and P solubilization—were the most effective inoculants. The combination of both functional traits was more effective than the separate application of biofertilizers with one trait only (**Figure 3**).

Impact of Biofertilizers by Crop Categories

Across all crop categories, the inoculation with biofertilizers showed an average yield increase by 16.2 ± 1.0% as compared to non-inoculated controls (**Figure 4A**). Yield response was

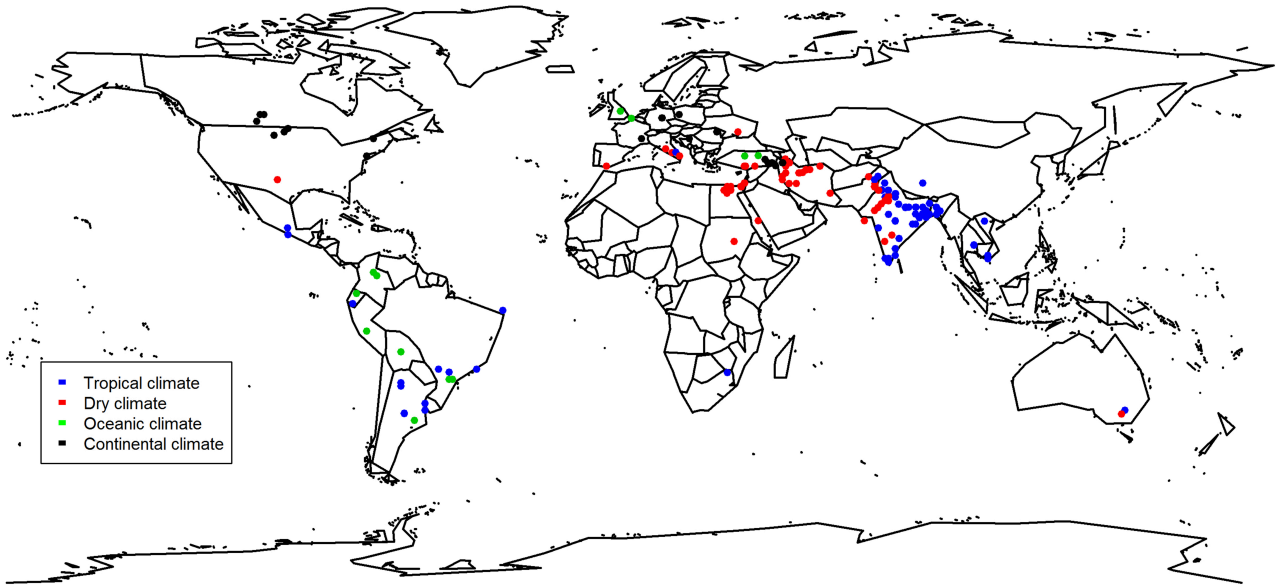

FIGURE 1 | Map showing origin of the study and their classification based on the climate. Some locations were not given by the study and were thus located with the name of the place given. Studies that were conducted under commercial conditions in the greenhouse are excluded from this map (Gravel et al., 2007; Luna et al., 2012; Bernabeu et al., 2015; all tomato).

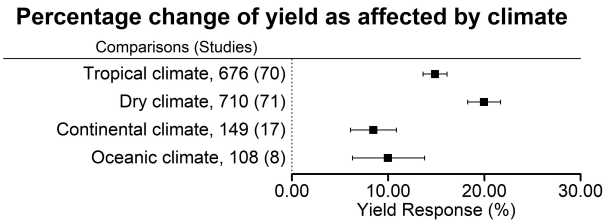

FIGURE 2 | Percentage change of yield in response to biofertilizer application as affected by climate. Mean values and 95% confidence intervals of the back-transformed response ratios are shown. There was a more pronounced effect in tropical and dry climates.

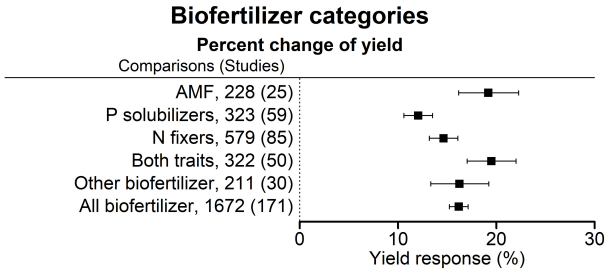

FIGURE 3 | Percentage change of yield in response to the application of various categories of biofertilizers. Mean values and 95% confidence intervals of the back-transformed response ratios are shown. There was a more pronounced effect with AMF and for N fixers in combination with P solubilizers.

distinctly lower for root crops than for all other crop categories, with legumes showing a tendency to superior response upon inoculation.

The overall improvement of PUE due to biofertilizers was 7.5 ± 0.8 kg yield per kg P (**Figure 4B**). PUE increase was most pronounced in legumes (7.8 ± 1.3 kg yield per kg P). Least improvement was found with root crops and the category other crops. On average NUE was improved by 5.8 ± 0.6 kg yield per kg N fertilizer through biofertilization (**Figure 4C**). Legumes manifested the highest response for NUE (8.3 ± 1.2 kg yield per kg N), root crops, vegetables, and the category other crops the lowest.

Response of Biofertilizers to Plant Available Phosphorus in Soil

Each crop plant, but even crop variety as well as microorganisms have an optimum level of abiotic factors for their physiology and growth. We tested the dependency of biofertilizers with regard to their induced effect size yield under different levels of plant available P, as P is a limiting element for plant growth in many regions of the world. Seven cohorts were formed with the level of plant available phosphorus in soil, which provided sufficient data for comparisons in each level and biofertilizer category. Our results indicate that AMFs have their optimum in yield increase at a low level of 15–25 kg P ha^{-1}. P solubilizing microorganisms have their best effect between 25 and 35 kg ha^{-1} soil available P (**Figure 5**). N fixers alone have an optimum in yield at more than 45 kg ha^{-1} available P; in combination with P solubilizers, this drops to 35–45 kg P ha^{-1} (**Figure 5**). In their optimum all biofertilizers except P solubilizers increase yield by more than 40%. In a meta-regression with the sum of soil available P and fertilizer P as an explanatory variable, the same increased

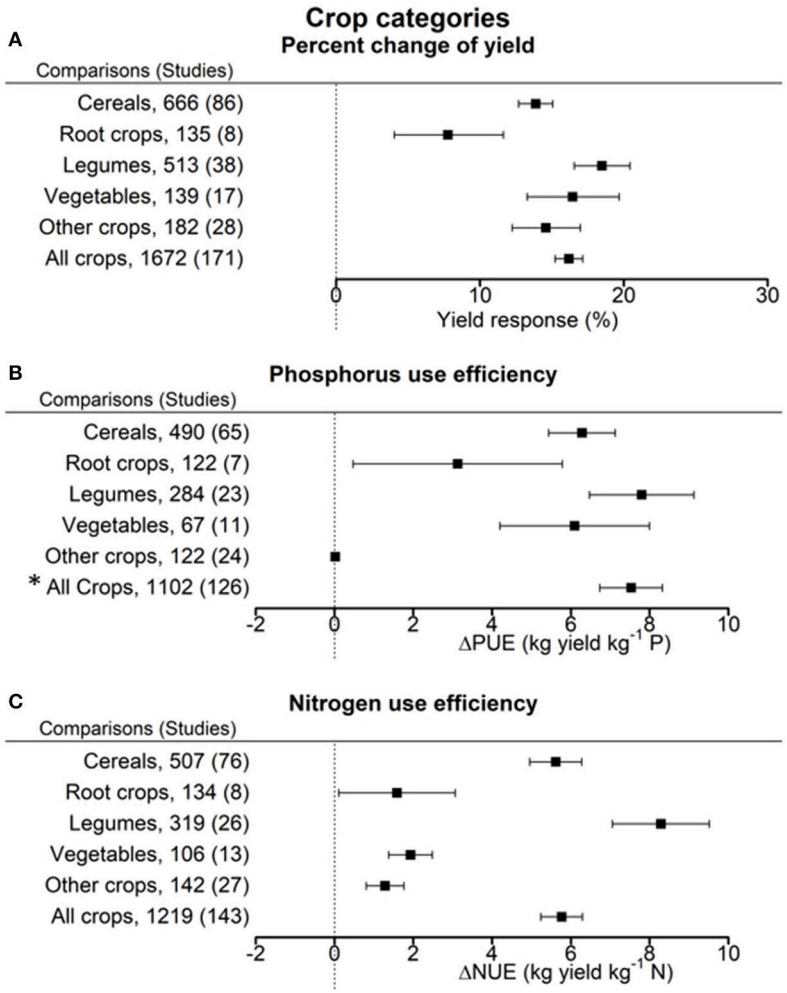

FIGURE 4 | Percentage change of yield **(A)**, change in phosphorus use efficiency (PUE) **(B),** and nitrogen use efficiency (NUE) **(C)** in response to biofertilizer application. Mean values and 95% confidence intervals of the back-transformed response ratios are shown. Yields of root crops were least responsive due to inoculation. PUE was improved in legumes, cereals and vegetables. NUE was improved in legumes and cereals but only to a minor extent in root crops and the other crops. *The high value for all crops is caused by the outlier calculation that resulted in different pairs being excluded for the full sample and the sub-samples.

efficiency at low P levels for AMF and the combined application of P solubilizers and N fixers was found (**Figure 6**). However, for P solubilizers and N fixers alone no relationship could be found.

Impact of Other Biofertilizers

We found a decrease in yield response for P solubilizers and even more for AMF with increased soil organic matter (**Figure 7**). We also identified pH as an important factor for the success of inoculation of AMF and as well for combined P solubilizers with N fixers (**Figure 8D**). With AMF there is a slight decrease in yield response at higher pH (**Figure 8C**).

Limitations

Meta-analyses face the problem of publication bias. Asymmetry in funnel plots can give information about a publication bias, but its interpretation is sometimes reported to be subjective (Terrin et al., 2005). Our statistical analyses of publication bias resulted in biases to both overly positive and overly negative results, but the bias identified is only moderate, and we thus refrained from adjusting the data to explicitly account for that but we refrain from further interpretation. Regarding variables of potential relevance that have not been covered, the initial soil microbial community had most probably an effect on the inoculation success. Some studies have reported initial populations of their inoculants in the soil, but information on this was too heterogeneous and scarce to be included in this analysis.

DISCUSSION

Are Biofertilizers a Viable Option for Dryland Agriculture?

Our results give strong indications that microbial inoculation is more successful in dry regions. The differences between dry and

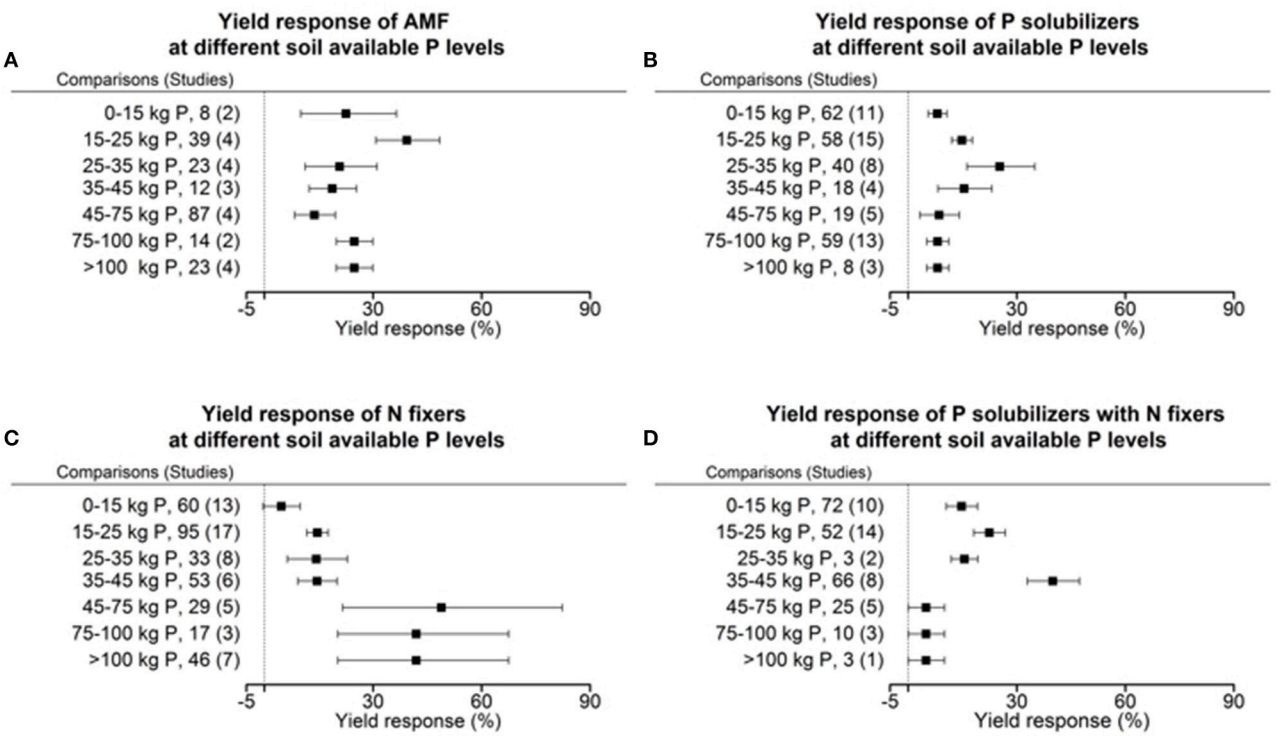

FIGURE 5 | Percentage change of yield in response to applications of AMF **(A)**, P solubilizers **(B)**, N fixers **(C)**, and N fixers in combination with P solubilizers **(D)** as affected by the levels of plant available phosphorus in soils. Mean values and 95% confidence intervals of the back-transformed response ratios are shown. Yield response of AMF is highest between 15 and 25 kg and with P solubilizers it is between 25 and 35 kg plant available P per hectare. Yield response in N fixers has its optimum within 45–100 kg and in combination with P solubilizers between 35 and 45 kg plant available P per hectare.

other climatic conditions are not necessarily thought to be based on microbes conferring drought resistance, but on differences of microbial community in the dry season. Yet microbes are also affected by soil fertility, which is usually lower in dry regions (Thomas et al., 2004). Especially soil organic matter (see **Table 4**) and soil nitrogen content are reduced. Accordingly, also organic P is lower in drier regions. Phosphorus is highly immobile in soil, particularly in dry soils with less water and less diffusion (Syers et al., 2008). This explains the stronger effect of biofertilizers and especially of P solubilizing bacteria and AMF under these conditions.

When dry soil is suddenly getting wet, there is a burst of availability of N and C, caused by lysis of microorganisms due to the rapid change in water availability (Kieft et al., 1987) and also by the release from non-microbial soil organic carbon (Appel, 1998). More N than C is mineralized which enables microbial degradation of materials with a low C:N ratio and results in further mineralization. This explains the commonly observed pulse of mineralization following wetting of dry and semidry soils (Bloem et al., 1992; Zaady et al., 1996; Cui and Caldwell, 1997; Austin et al., 2004). Both events explain the increased yield effect of biofertilizers under dry climate: Biofertilizers immobilize N to make it available later or directly improve the uptake by plants by facilitating the conversion of ammonium to nitrate and are able to prevent gaseous losses of nitrogen. Other released nutrients may as well be taken up by microbial inoculants and then become plant available later in the season.

Secondly, dry regions are, even with irrigation, still dryer compared to humid areas and often also hotter, causing more evapotranspiration from plants and soil. Biofertilizers like *Azospirillum* may release phytohormones like auxin which enhance root branching and also root elongation. This would be a clear advantage for plants in dry areas (Dobbelaere et al., 1999; Steenhoudt and Vandereyden, 2000). Furthermore, biofertilizers are able to produce other plant hormones like gibberellins and cytokinins in the case of *Azotobacter* (Bhardwaj et al., 2014) reducing stress in the plants and stabilizing their yields. Some bacteria produce ACC deaminase and some biofertilizers are specifically selected for their ability to do so. In stress situations, like drought, plants produce ethylene, which reduces plant growth and may also limit nodulation in leguminous plants. ACC deaminase producing bacteria are able to degrade ethylene thus allowing the plants to grow better by reducing the impact of signal molecules (Shaharoona et al., 2007). Also proline, which accumulates as a common physiological response to various stresses, is degraded by bacteria and improves drought resistance under modest drought (Straub et al., 1997; Verbruggen and Hermans, 2008). This effect was also proven to be agronomically important for plants under drought (Naseem and Bano, 2014; Kumar et al., 2016). Stress situations are more likely in dry

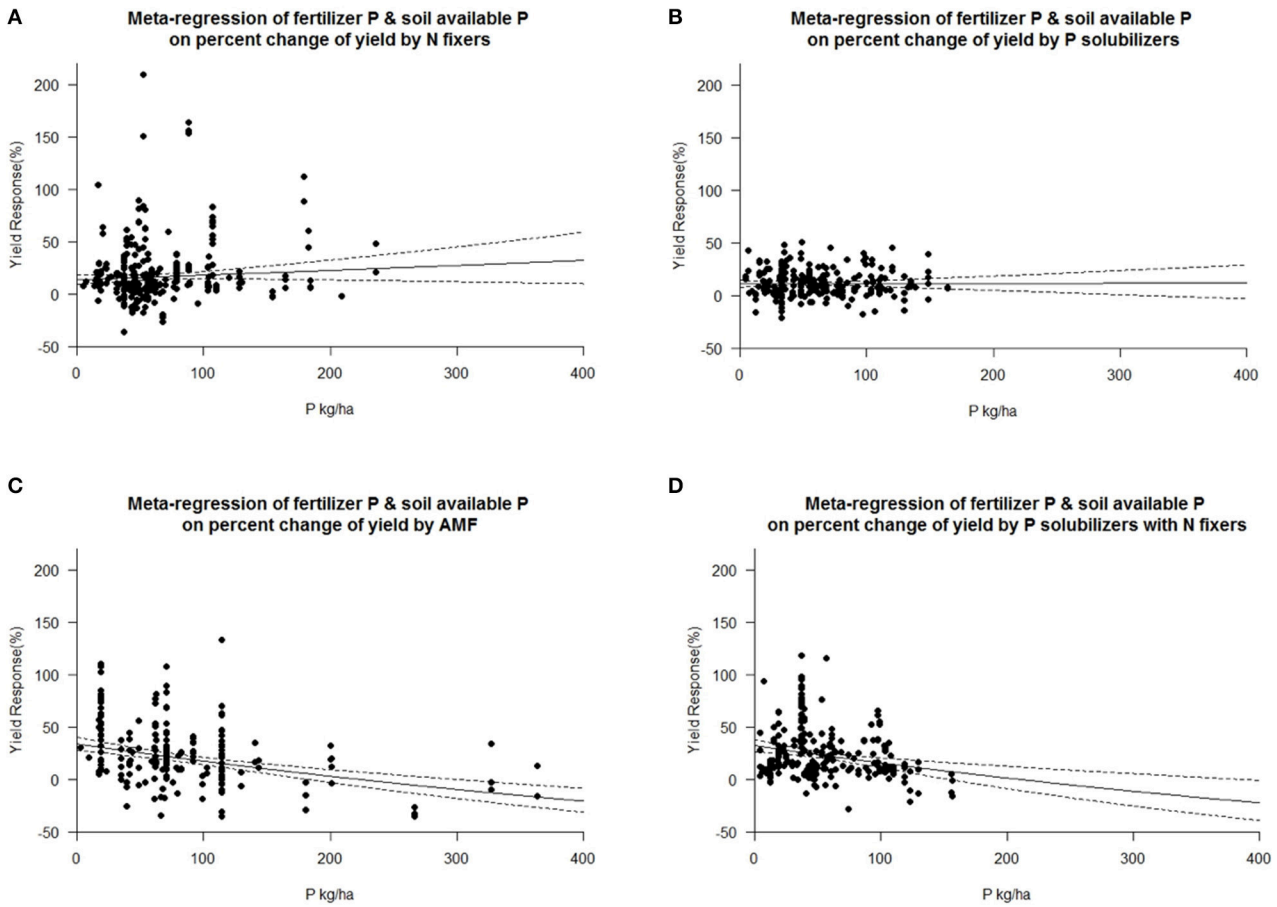

FIGURE 6 | Mixed effects model with fertilizer P added to soil available P as moderator for various biofertilizer categories. Dotted lines depict the confidence interval. **(A)**, $n = 316$, $R^2 = 0.08\%$, $p = 0.1783$ **(B)**, $n = 255$, $R^2 = 0\%$, $p = 0.9438$; **(C)**, $n = 195$, $R^2 = 18.74\%$, $p = <0.0001$; **(D)**, $n = 230$, $R^2 = 5.47\%$, $p = 0.0002$.

regions where also salinity and nutrient deficiencies limit plant growth.

What Are the Best Biofertilizers?

Our meta-analysis reveals that AMF and combined application of P solubilizers and N fixers are the best inoculants. The higher yield increases by the combinations of the two functional traits N fixation and P solubilization than their separate application suggests an absence of competition and rather synergies between the two traits. Similar numbers for yield increase after inoculation with AMF were found by Lekberg and Koide (2005), who analyzed 290 glasshouse and field trials in a meta-analysis. Berruti et al. (2016) found in their meta-analysis that both yield and plant nutrition were significantly improved by inoculation with AMF under open field conditions in 92% of 112 experiments. In the literature, some microorganisms with the ability to fix nitrogen have been shown to contribute only to a small extent to the N nutrition of crops, and that these results are highly variable (Lee et al., 1994; Bremer et al., 1995; Santi et al., 2013). Our results indicate that their contribution to yield is substantial and with low variation (**Figure 3**).

Furthermore, a certain amount of plant available P is necessary for all of the biofertilizer groups and none had their optimum at the lowest cohort between 0 and 15 kg ha^{-1} soil available P. In AMF with the best growth promotion at a low level, the growth promotion is well known to depend on the P status of the plant (Smith and Read, 2008a). AMF are able to access phosphorus in soil pores, too small for plant roots, and also extend the access to P in distant soil patches through their hyphal network (Smith and Read, 2008b). Lekberg and Koide (2005) found a greater potential for growth responses in soils with low levels of plant available P in soil, however variability was high. N fixation has large requirements of P and the need is satisfied only at higher levels of P (Graham and Vance, 2000). Leguminous plants for example have developed P solubilizing strategies themselves to satisfy the need of their symbionts. In the meta-analysis by Augusto et al. (2013) it was shown that P availability drives plant growth and also biological nitrogen fixation which explains the strong response at high levels of plant available P in soil in our study. In a meta-regression we have tested furthermore whether our results achieved with soil available P is also found when taking the sum of soil available P and fertilizer P as the explanatory variable. However we found

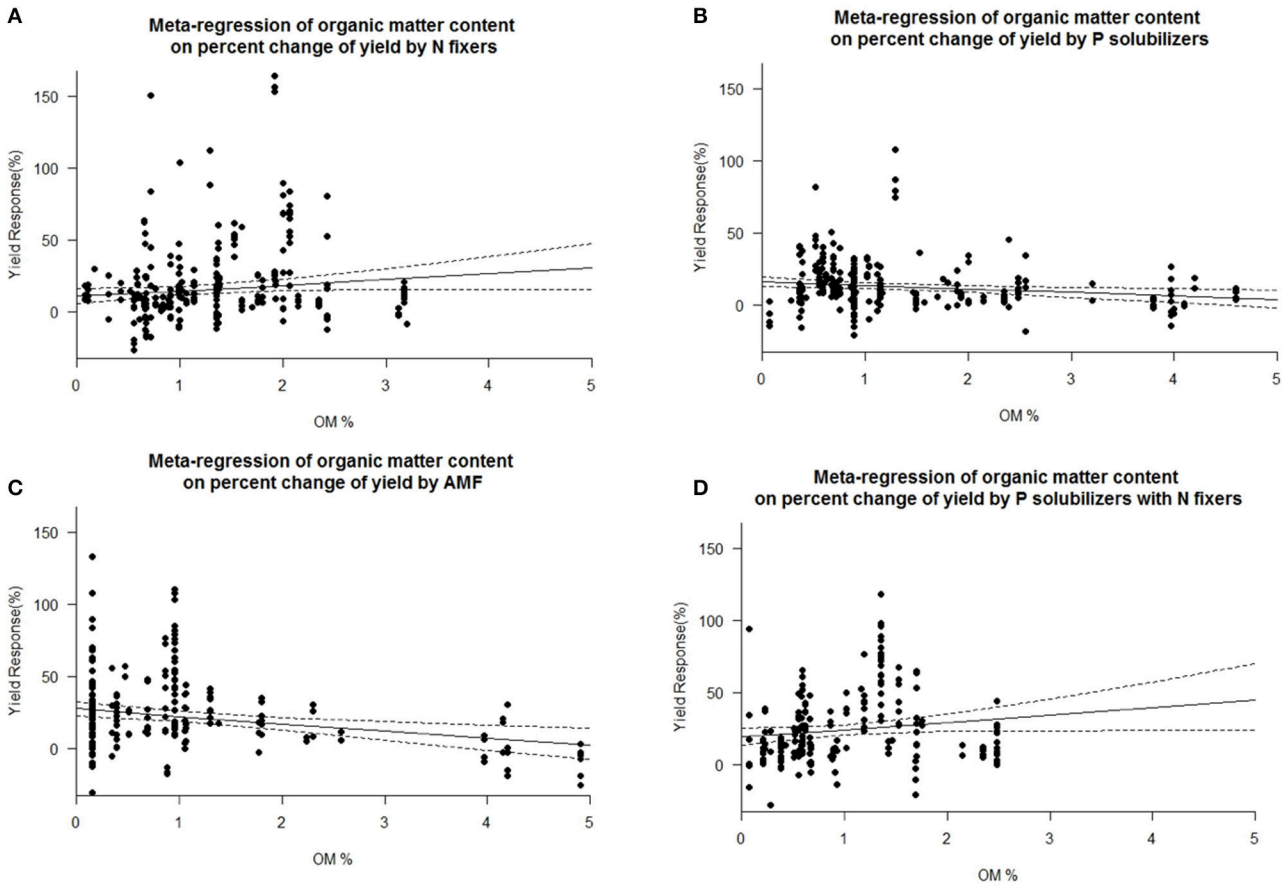

FIGURE 7 | Mixed effects model with organic matter (OM) as moderator for various biofertilizer categories. Dotted lines depict the confidence interval. **(A)**, $n = 313$, $R^2 = 0.0\%$, $p = 0.9174$; **(B)**, $n = 251$, $R^2 = 1.96\%$, $p = 0.0063$; **(C)**, $n = 202$, $R^2 = 4.8\%$, $p = 0.0007$; **(D)**, $n = 207$, $R^2 = 2.04\%$, $p = 0.0492$.

that to result in less of an explanations than before. Considering that only 10–20% of P contained in the crop originates from the most recent fertilization and the remaining 90–80% comes from the reserves accumulated in the soil in earlier fertilizer applications (Sharpley, 1986; McLaughlin et al., 1988), it is no surprise that plant available P in soil is a better control variable.

We are aware of the fact that many biofertilizers may have multiple functions and traits, although not specified by the producers, or by the researchers. Nonetheless we categorized the inoculants to the best of our knowledge. Many studies have used combinations of different biofertilizers and synergistic effects cannot be excluded. Some biofertilizers can fix nitrogen while also solubilizing phosphorus, but they were selected for other traits as well e.g., plant hormone production, solubilization of other nutrients such as Zn or Fe or plant defense [antibiotic 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN)]. However, in a separate analysis we found no general superiority to mono inoculation (multi inoculation 15.5 ± 1.4% vs. mono inoculation 16.9 ± 1.3% yield increase). P solubilizers and AMF are most successful at the low levels of plant available P prevalent in soils of tropical regions. Biofertilizers were best in both dry and humid tropics. We also found a decrease in yield response for P solubilizers and even more for AMF with increased soil organic matter (**Figure 7**), which is likely caused by an increased microbial activity, making it difficult for new microorganisms to establish (Schnürer et al., 1985; Paul, 2016). Also soil organic matter contains organic phosphorus in microbial biomass and other organic pools. We also identified pH as an important factor for the success of inoculation of AMF and as well for combined P solubilizers with N fixers (**Figure 8D**). Under low and high pH macronutrients are less available for plants. Our results indicate that AMF make only accessable macronutrients at neutral pH more available. Combined P solubilizers and N fixers are effective at high pH. However P solubilizers and N fixers applied alone are independent of pH. With AMF we even found a slight decrease in yield response at higher pH (**Figure 8C**), which again corresponds to less soluble macronutrients and especially nitrogen and phosphorus.

There is circumstantial evidence why legumes were most responsive to biofertilizers across all effect sizes. Biofertilizers applied to legumes consisted in 12% of all included studies of rhizobia, which were selected to build compatible symbioses with

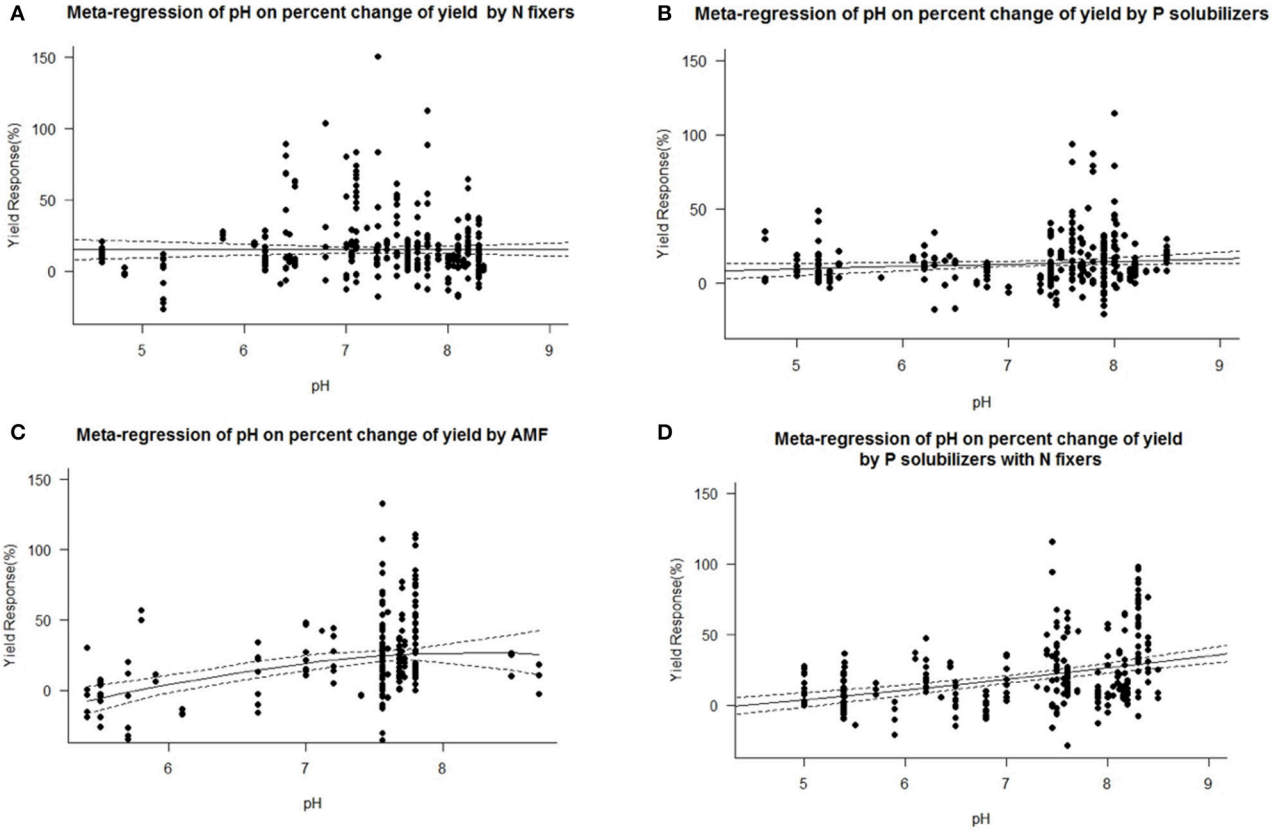

FIGURE 8 | Mixed effects model with pH as moderator for various biofertilizer categories. Dotted lines depict the confidence interval. **(A)**, $n = 450$, $R^2 = 0.35\%$, $p = 0.2864$; **(B)**, $n = 294$, $R^2 = 1.19\%$, $p = 0.0405$; **(C)**, $n = 206$, $R^2 = 14.22\%$, $p \leq 0.0001$; **(D)**, $n = 228$, $R^2 = 13.57\%$, $p \leq 0.0001$.

their host plants, but rhizobial inoculum is already present in many soils anyways. Legumes have evolved specific symbioses with N fixing rhizobia but require also other nutrients; reportedly the phosphorus requirement of nodules is up to three times higher than the needs of the surrounding roots (Vadez et al., 1997). Other microorganisms or biofertilizers may help to fulfill this additional nutrient need. In fact, legumes were shown to benefit by an additional AMF inoculation (Mortimer et al., 2008; Omirou et al., 2016). The applied biofertilizers, often with multiple traits such as N fixation and P solubilization, seem to act more synergistically in legumes than in other plants. Interestingly the addition of extra microbial inoculants to sole rhizobia treatments alone improved crop yield also in the range of 19.2% (mean of 59 comparisons from 12 studies), substantiating the synergistic effect between N fixers and P solubilizers.

CONCLUSIONS

We have analyzed three different effect sizes each giving a different perspective on the success of biofertilizers. It was found that dryland agriculture can benefit most from biofertilizers. Due to climate change, in the future there will be even more dryland areas globally. Biofertilizers are thus a promising option for sustainable agriculture. In the future, pretests of the soil community may predict the competitive chance of biofertilizer in a specific soil and help to efficiently produce adapted biofertilizers for each specific application.

AUTHOR CONTRIBUTIONS

LS, AG, PM, TB, MM, AM, and NM: Designed research; LS: Performed research and analyzed data; LS, PM, AM, TB, and NM: Wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02204/full#supplementary-material

Figure S1 | Prisma flow diagram of the literature search.

Figure S2 | Funnel plots of the change in yield of **(A)** tropical climate **(B)** dry climate **(C)** continental climate **(D)** oceanic climate. Mean difference of yield on the horizontal axis is plotted against their corresponding standard errors (SE) on the vertical axis.

Supplementary Data Sheet S1 | Data used for meta-analysis.

Supplementary Data Sheet S2 | Study list used for meta-analysis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplement

PRISMA 2009 Flow Diagram

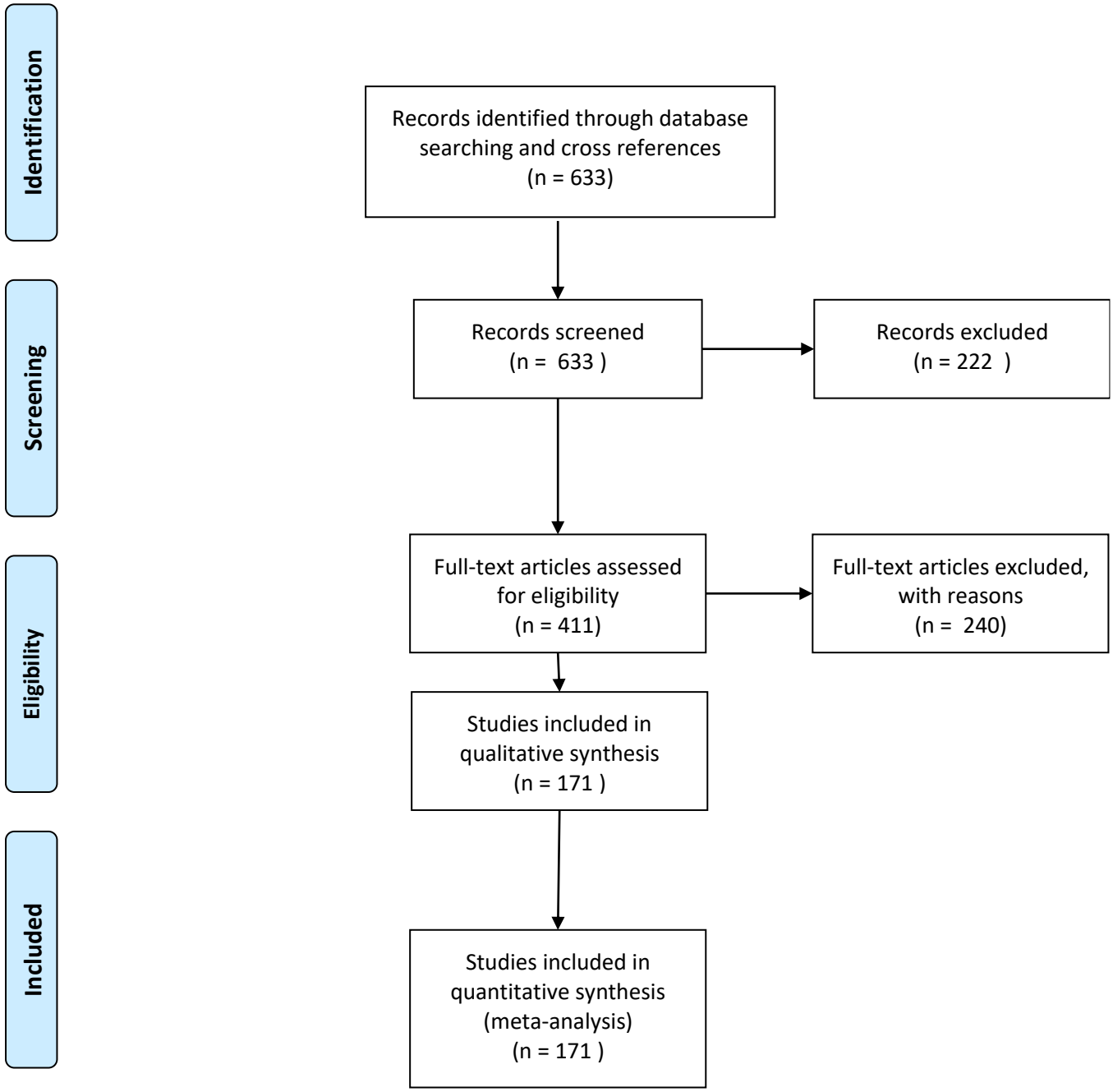

Figure S1: Prisma flow diagram of the literature search.

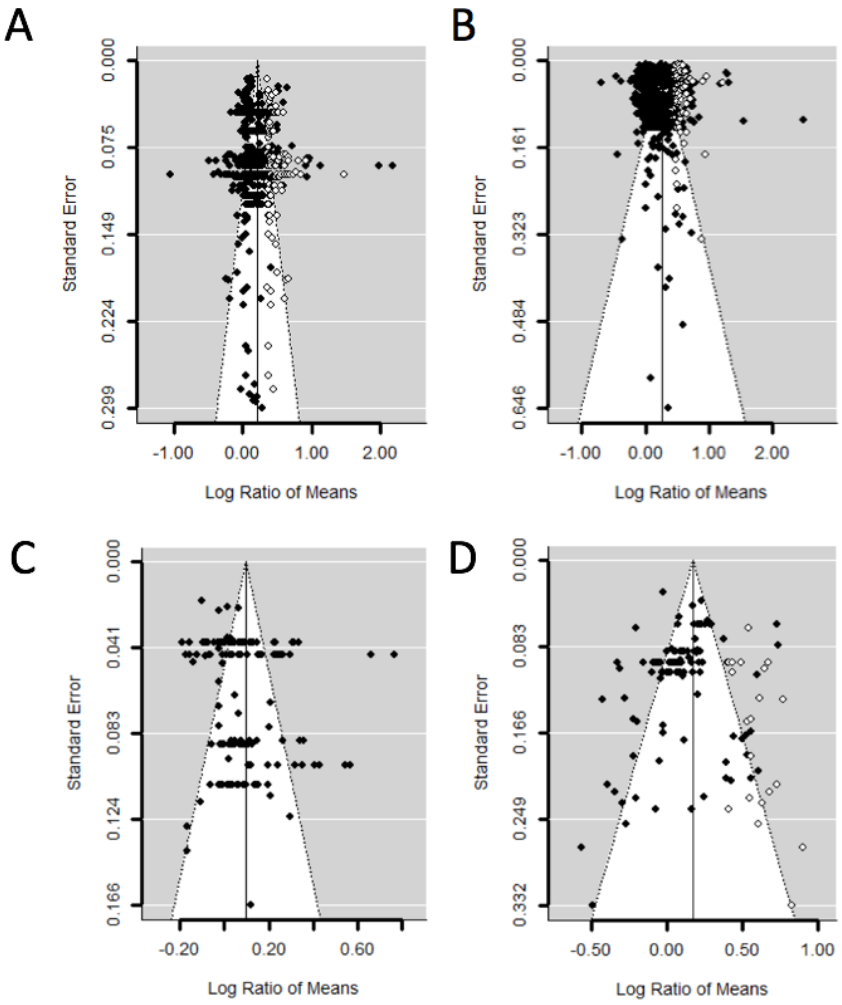

Figure S2: Funnel plots of the change in yield of A) tropical climate B) dry climate C) continental climate D) oceanic climate; mean difference of yield on the horizontal axis is plotted against their corresponding standard errors (SE) on the vertical axis.

The following study has been conducted by me, Lukas Schütz, and I am the main author. It is in preparation to be submitted to a peer reviewed journal.

Growth promotion of intercropped pigeon pea (*Cajanus cajan*) and finger millet (*Eleusine coracana*) by arbuscular mycorrhizal fungi and hyphal spread

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Abstract

Arbuscular mycorrhizal fungi (AMF) can substantially contribute to a more resilient, sustainably intensified dryland farming system. We are interested in the possibility to use AMF as "biofertilizers" in an intercropping system in Indian agriculture, planting pigeon pea (*Cajanus cajan*) seedlings pre-inoculated with AMF into a field sown with finger millet (*Eleusine coracana*).

To study the potential of the AMF to spread from AMF-inoculated pigeon pea to un-inoculated finger millet seedlings, we established experimental microcosms in the greenhouse, in which one pigeon pea and two finger millet plantlets were kept in separate pots, connected by soil bridges of 5 or 12 cm length, inaccessible to roots but accessible to fungal hyphae. The pigeon pea plants were pre-inoculated with *Claroideoglomus etunicatum, Rhizophagus fasciculatus* or *Rhizophagus irregularis.*

By destructive sampling we estimated a hyphal growth of 4.1mm d^{-1} by *C. etunicatum* crossing 12cm. The biomass of pigeon pea was more than doubled with all AMF species. In the short microcosm with *R. fasciculatus* P-content per plant (+72.5%) and dry panicle weight (+81.4%) of the more distant finger millet was significantly increased. With *R. irregularis* the dry biomass and dry panicle weight of the closer finger millet plant was significantly decreased (-45.8% and -38.6%). With *C. etunicatum* hyphal length density of *R. fasciculatus* was lowest, but not significant. Surprisingly, in the long microcosm, the third species, *C. etunicatum*, promoted growth of the more distant finger millet more than the other inoculants. Furthermore by applying ^{15}N isotopes to pigeon pea we revealed that in both lengths of the microcosm *R. fasciculatus* and *C. etunicatum* transported nitrogen from pigeon pea to finger millet across distances of up to 12cm but *R. irregularis* did not.

We found that AMF hyphae could spread readily through the soil bridges from the roots of pigeon pea to the roots of finger millet, covering distances of up to 35 cm in 20 weeks, and have growth promoting effects there. These distances are relevant for the row-wise planting of crops and we conclude that the row distance between the crops and the choice of AMF species plays a crucial role for the application of AMF as biofertilizer.

Keywords: Biofertilizer, Intercropping, Pigeon pea (*Cajanus cajan*), Finger millet (*Eleusine coracana*), Hyphal spread, Arbuscular mycorrhizal fungi (AMF), Compartment

Introduction

Mixed cropping and intercropping describe the planting of two or more different crop species or genotypes at the same time or with a temporal overlap which is called relay-cropping. This practice increases the diversity of crops in agriculture but also biodiversity (Brooker et al. 2015) At the same time yields can be increased when compared to mono-cropping of each crop alone on the same area of land. Such yield increase is called overyielding. In this mixture interspecific plant interactions change the use of resources (Vandermeer 1989; Zhang and Li 2003). Resources are either used complementarily and nutrients are used which in monocropping would not be utilized. Complementary use of resources can happen through various mechanisms like complementary root architecture which enables the plant combination to make the best use of space for taking up nutrients and water from all soil layers. Or one crop facilitates the uptake of a nutrient for the other by increasing the pool of nutrients. Facilitation can happen in all combinations with legumes when some of the fixed nitrogen by rhizobial symbiosis is made available to the other crop (Hauggaard-Nielsen and Jensen 2005; Li et al. 2007). Mixed cropping is often discussed to be especially suited for marginal lands (Qiao et al. 2015). It can also act as a buffer against extreme events when one crop is more resilient than the other which stabilizes yields over time.

In the soil mutualistic root organisms like arbuscular mycorrhizal fungi (AMF) can substantially contribute to a more resilient, sustainably intensified dryland farming system. Inoculating crops with AMF via application to seed furrows or by coating seeds can increase yields (Lekberg and Koide 2005), and yield responses have been shown to be higher in dry climate and at low available Phosphorus (P) content (see chapter 1 of this thesis). We are interested to study the possibility to use AMF as “biofertilizers” in an intercropping system in Indian agriculture, planting pigeon pea (*Cajanus cajan*) seedlings pre-inoculated with AMF into a field sown with finger millet (*Eleusine coracana*).

With the combination of these three elements including plant diversity and soil organisms we want to make optimal use of their ecological traits and utilize the functional diversity to establish a more sustainable agricultural system with better use of resources. Pigeon pea is a deep rooting legume with rhizobial nitrogen fixation. Finger millet on the other hand is a shallow rooted cereal with C4 carbon assimilation. Pigeon pea is generally a popular crop used for mixed cropping with 65 mixtures recorded in India (Ahlawat et al. 2005). There is also a temporal complementarity between the two as finger millet is harvested earlier, hence the peak of nutrient uptake is shifted. If pigeon pea is grown in a nursery before the onset of the monsoon and the regular planting time, they have the advantage to be larger at time of flowering thereby having higher yields (Praharaj et al. 2015). The success though depends on the onset of the monsoon and planting time (Pavan et al. 2011). The pregrowth in a nursery is also ideal for the inoculation with AMF, ensuring the preferential colonization with the best-suited preselected AMF strain.

To study the potential of the AMF to spread from AMF-inoculated pigeon pea to uninoculated finger millet seedlings, we established experimental microcosms in the greenhouse, in which one pigeon pea and two finger millet plantlets were grown in three separate pots, connected in series by two soil bridges of 5 or 12 cm length. These soil bridges were made inaccessible to roots but accessible to fungal hyphae by separating them from the pots with a fine nylon screen (mesh size 21μm). Hyphal growth is stimulated by signalling molecules like strigolactones (Akiyama and Hayashi 2006) which guide the hyphae to the next plants. By including activated carbon in the hyphal compartment we hope to learn more of how directed the hyphae grow. Thus, several questions are addressed with this study:

I. Does AMF inoculation enhance the growth of pigeon pea?
II. Does single inoculation with AMF on one pigeon pea have growth promoting effects on subsequent and younger finger millet plants as well?
III. How do AMF species differ in their ability to connect with finger millet plants and how fast do they grow?
IV. Are AMF involved in the transport of nitrogen and how far can they transport it?
V. Are the hyphae, also over longer distances, guided by signalling molecules?

Material and Methods

Experiment 1: Characterization of different AMF species for their hyphal growth, nitrogen transport and growth promotion of finger millet and pigeon pea

Experimental setup: The microcosms in experiment 1

The experimental setup of the "microcosms" with one pigeon pea and two finger millet plants is shown in Fig. 1. The finger millet plant closest to pigeon pea is hereafter referred to as finger millet 1 or FM1 and the more distant one as finger millet 2 or FM2. Each pot was separated from the interconnected hyphal compartment (HC) by a nylon mesh (pore size 21 µm) and a fiber glass mesh with a larger pore size to stabilize the nylon mesh. It was fixed by a ring-adapter fitting tightly into the tube. Whenever long HC (12 cm) were used (Fig. 1A) these microcosms were termed long microcosm and whenever short HC (5 cm) they were termed short microcosms. Containers were washed with detergent and rinsed with tap water. Then they were sterilized by spraying with 70% ethanol before they were filled with substrate.

Three AMF species were tested: *Claroideoglomus etunicatum, Rhizophagus fasciculatus* and *Rhizophagus irregularis*. Additionally, *C. etunicatum* and *R. fasciculatus* were grown in a long microcosm with no mesh separating the roots of the plants. A non-mycorrhizal control was set up for both microcosms. All treatments consisted of four replicates (Table 1). Pots were randomized weekly.

Table 1: Experimental Design of experiment 1 (n=4).

AMF species	Experimental setup
Claroideoglomus etunicatum	Short Microcosm
	Long Microcosm
	Long Microcosm without separating mesh
Rhizophagus fasciculatus	Short Microcosm
	Long Microcosm
	Long Microcosm without separating mesh
Rhizophagus irregularis	Short Microcosm
	Long Microcosm

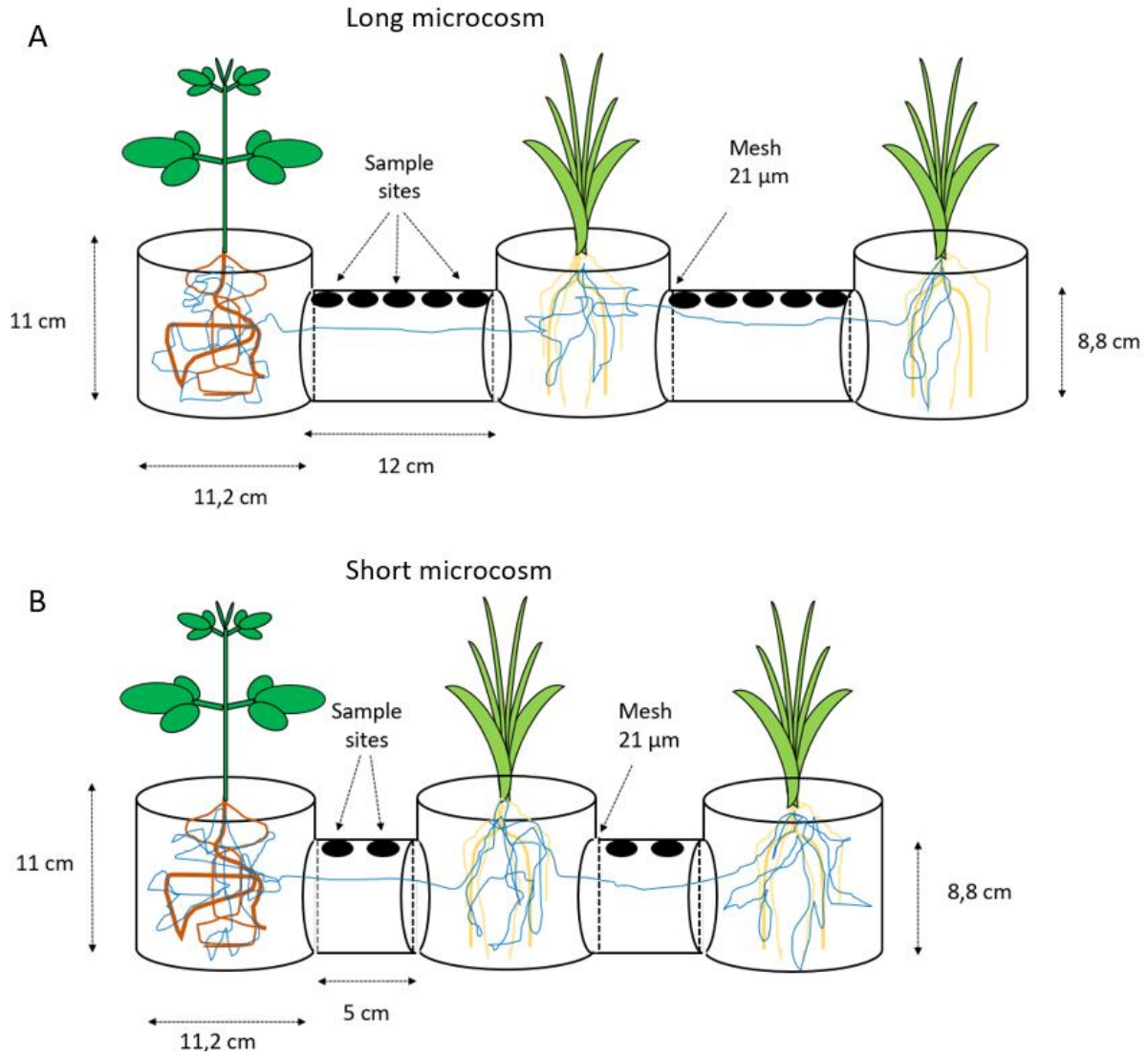

Figure 1: Experimental design of experiment 1. (A) depicts the long microcosm with an HC of 12 cm and (B) the short microcosm with an HC of 5 cm. On the left pigeon pea and on the right plants of finger millet. Blue lines represent fungal hyphae at an advanced stage of the experiment where they have already colonized both finger millet plants.

Substrate

The growth substrate consisted of Sorbix US Premium oilbinder (Chem-Sorb) (Maagtechnic AG, 8600 Dübendorf, Switzerland), quartz sand (0.6 – 1.2 mm, Alsace, Kaltenhouse, Trafor AG Basel) and sieved (<3mm) Loess (Biel-Benken, Switzerland) in a mixture of 1:4:1. Loess was autoclaved with 50ml water per 5 kg. Quartz Sand and Chem- Sorb was heated for 9h at 80°C for sterilization. Nutrient content of the whole mixture was 101 mg P/kg, 200 mg K/kg, 331 mg Mg/kg (all three analyzed via ICP-OES after extraction with nitric acid), 9.58 mg nitrate/kg (water extract and analyzed with UV/Vis spectroscopy), pH 6.3 (Umweltanalysezentrum, Salucor GmbH, Filderstadt, Germany).

Plants

Seeds of pigeon pea and finger millet originating from India belong to the variety TTB7 and GPU28 respectively (Ankur Seeds Pvt. Ltd, Bangalore, India). Seeds were surface-sterilized by soaking them 30 s in ethanol 96% and 2 min in NaClO 5% (commercial bleach), then washed by 0.01N HCl and washed 8 times with sterile water (Somasegaran and Hoben 1985). Then three seeds were placed into one pot together with 5 g of the fungal inoculant, containing 15 spores/g. After 10 days the strongest seedling was selected and the other two discarded. Finger millet seeds were pre-germinated in vermiculite and covered by sand. Seedlings of a similar size were selected for transplanting into the experimental pots.

Inoculants

Three species of AMF were tested: *Rhizophagus fasciculatus* (Thaxt.) C. Walker & Schuessler (formerly *Glomus fasciculatum* Gerdemann & Trappe), *Claroideoglomus etunicatum* C. Walker & Schuessler (formerly *Glomus etunicatum* W.N. Becker & Gerd) and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & Schuessler (formerly *Glomus intraradices* N.C. Schenck & G.S. Sm.), strain BEG75.

AMF species were maintained in pot cultures with leek as a host plant. Spore numbers were counted after they were isolated with a sugar gradient (Talukdar and Germida 1993). Inoculum of *Claroideoglomus etunicatum* had 132 spores/g, *Rhizophagus fasciculatus* 53 spores/g and *Rhizophagus irregularis* BEG 75 15 spores/g. Inoculum of *C. etunicatum* and *R. fasciculatus* was diluted to 15 spores/g.

Microbial wash was obtained by wet sieving 100 g of inoculum with 1 L of water through a 32µm sieve and through a folded filter (Schleicher and Schuell, LS 14 ½). Amount of washed inoculum was partitioned by the three species relative to the amount applied to each treatment. 10 ml of the microbial wash were added to each control microcosm (Koide and Elliott 1989).

Pigeon pea was inoculated with *Bradyrhizobium* sp. strain IHP 195, DSM No. 5969 which was grown on yeast mannitol (YM) Agar plates for 5 days and then transferred into liquid YM Medium. This culture was pipetted to the pigeon pea plants with a CFU of 10^6.

Growth, sampling and harvest

Pigeon peas were sown with three seeds per pot, thinned to one seedling per pot, and grown for 30 days in their compartment to establish symbiosis. The sample sites were covered by aluminum foil. Then the hyphal compartment was filled with substrate as well as the compartments of finger millet, and seedlings (7d old) of finger millet were transplanted into the

finger millet compartments. Plants were watered every two days to 39% of field capacity (FC 384mL/Kg substrate) without any signs of water stress. Watering was regularly adjusted by measuring the evapotranspiration. The plants were grown in the greenhouse of the University of Basel under controlled conditions with 16 h light at 25°C-35°C and 220 µE m-2 s-1, 8 h dark at 20°C, and constant relative humidity of 65%. After 14 days first soil core samples (core diameter 13mm) were taken at the first sampling hole (3 mm distance). The second sampling was taken after 28 days at 30mm in the short microcosm and at 54 mm in the long microcosm. A third sampling was only conducted in the long microcosm at 102 mm. At the same time, the length of the finger millet plants were recorded. Six weeks after transplanting finger millets 5 ml of a full strength Hoagland's solution without $NH_4H_2PO_4$ was applied to pigeon pea (Gamborg and Wetter 1975). Additionally, powdered 100mg $FePO_4$ was applied together with the ^{15}N isotopes (see below for further details) and constantly stirred during the application. $FePO_4$ was applied as the only P source as it is known that pigeon pea can mobilize phosphate from hardly soluble forms by way of root exudates (Ae et al. 1990; Shibata and Yano 2003). Plants were harvested 23 weeks after pigeon pea was sown, and nodulation was assessed visually. Shoots and roots were separated, dried for 22h at 105°C, and ground to a fine powder at 30Hz using a mixer mill (MM2224, Retsch, Haan, Germany) for subsequent P and 15N analysis.

Application of ^{15}N

Six weeks after the finger millet seedlings were transplanted ^{15}N was applied to the soil around the pigeon pea plant. 4g of $^{15}NH_4{}^{15}NO_3$ (Cambridge Isotope Laboratories) were dissolved in 4L water, 4g of $FePO_4$ was added and the mixture constantly stirred. 100mL containing 100mg $^{15}NH_4{}^{15}NO_3$ and 100mg of $FePO_4$ was then applied to each pigeon pea. Thereafter the plant were watered to ensure infiltration of ^{15}N, however it was ensured that nothing leaked out.

Hyphal length measurement

Soil cores were sieved through a 500 µm and a 32 µm sieve. The resulting material in the 32 µm was homogenized with 100 ml H_2O in a blender. The suspension was then diluted to a total of 500 ml and stirred. After stop of stirring subsamples of 1 ml were taken after 10, 20, 30, 40 and 50 seconds 2 cm below the surface. Those five subsamples were combined into one. This sample was then placed on a membrane filter with a millimeter grid and water was sucked. The wall of the filtration unit was washed with water and the filter was stained with 1ml trypan blue for 2 minutes. Under the microscope (x200) all hyphae were counted, which intersected with

crosses on the grid (Newman 1965; Sylvia 1992; Thingstrup et al. 2000) and hyphal length density (HLD) was calculated per g of dry soil.

Analysis of root colonization

Root samples were taken at harvest. The roots were washed and cut into pieces of 1cm. They were then bleached and stored in 10% (w/v) KOH at 4°C and in the case of pigeon pea afterwards heated for 8 min at 90°C in a water bath. They were then stained with trypan blue (0.05% lactic acid, glycerol, water 1:1:1) for 15 min at room temperature. After destaining in water they were examined for possible colonization of AMF (Phillips and Hayman 1970). Proportion of roots colonized by AM hyphae, arbuscules and vesicles was calculated after Brundrett and McGonigle 1994, examining 100 intersections on 25 randomly chosen root pieces for each root sample.

Phosphorus Analysis

P-content of shoots and roots was measured using the molybdate blue method on a Shimadzu UV-160 spectrophotometer (Shimadzu Biotech, Duisburg, Germany) after acid digestion (Murphy and Riley 1962).

Nitrogen isotope analysis

The ^{15}N content and total N content of plants was analyzed with isotope ratio mass spectrometry (Delta V Plus, Thermo Fisher Scientific, Germany). Relative ^{15}N uptake was calculated by dividing the uptake of individual plants by the total uptake of both plants of the microcosm.

Experiment 2: AMF hyphal spread and active carbon as an inhibitor of directed hyphal growth

Experimental setup

A second experiment was conducted to analyze the hyphal growth of *Claroideoglomus etunicatum* in detail which had fast growing hyphae with the best growth promotion of finger millet. Three seeds of pigeon pea were sown. In 5 systems no germination occurred after one week of sowing and seedlings were carefully transplanted to these pots. They were then thinned to one plant per system after two weeks. As previously the pigeon pea was inoculated and grown for one month to establish symbiosis. Then HC (12 cm) was attached and one FM compartment was attached (Fig 2). The pieces were taped with a transparent tape to control visually for roots

entering the HC across the ring connecting the compartments. FM were sown as five seeds per pot and thinned to one seedling 15 days after sowing. Hyphae were sampled destructively two weeks, three weeks, four weeks, five weeks and eight weeks after the microcosms were joined, similar to the study by Hart and Reader (Hart and Reader 2005). Each sampling was replicated 5 times.

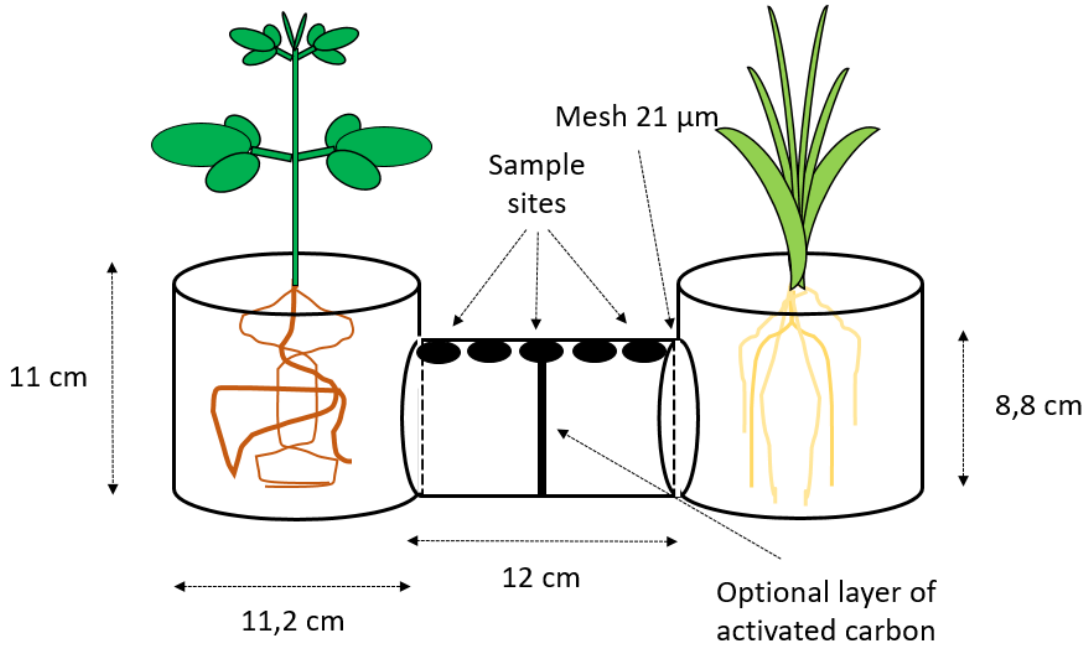

Figure 2: Experimental design of experiment 2. On the left pigeon pea and on the right plants of finger millet.

In this experiment it was furthermore tested whether hyphal growth is influenced by a layer of activated carbon (AC) (Merck, Art. Nr. 2186). One set of 5 replicates was equipped with such a layer and inoculated pigeon pea plants. No control with a layer of AC and non-inoculated pigeon pea plants was added. Thus comparisons with the control treatments without AC are not possible because AC may have an effect on the plants also without AMF. For any conclusions the debatable precondition is that such influence does not exist. A layer of 7g of AC (Fig S9) was applied at the centre of the hyphal compartment while the substrate was filled in a vertical position of the microcosm to establish an even layer. HLD in the systems with AC was only sampled at the two sample sites on both ends.
Substrate, plant varieties, *Bradyrhizobium* strain were the same as in experiment 1. No fertilization was applied in this experiment.

At each sampling the systems and plants were harvested destructively. All 5 soil cores in systems without AC and two soil cores in systems with AC were extracted using a core borer

(core diameter 12mm), samples to estimate root colonization of AMF were taken and shoot length and fresh biomass was measured. All methods were the same as in experiment one. Only another mixer was used for separating and cutting the hyphae (Philips ProBlend4, 1.5L 400W). The plants were grown in the greenhouse of the University of Basel under controlled conditions with 16 h light at 25°C-35°C and 220 µE m-2 s-1, 8 h dark at 20°C, and constant relative humidity of 65%.

Statistics

SPSS (v20) was used for statistical analysis of experiment 1. All treatments and parameters were tested for normal distribution before using other statistical methods. One-way ANOVA and Tukey test was used for post-hoc analysis to identify significant differences between the plant's response to the inoculants at a significance level of $p < 0.05$. Interactions between factors were tested for the two sizes of the microcosm and the different inoculants with a two-way ANOVA.

Hyphal growth per day was estimated with the package "Scatterplot3D" (Ligges and Mächler 2003) in the R Software Version 3.2.3 and the interface R-Studio Version 0.99.491. A linear regression plane was calculated for Hyphal length density as dependent on sampling time and distance from pigeon pea and the speed of growth calculated from the resulting formula. Results are presented as mean value and standard error (SEM) for each individual treatment.

Results

Influencing factors on hyphal growth and growth promotion

Data for biomass of the plants at the final harvest are shown in Fig.2. Two replicates of the control in the long microcosm were colonized by mycorrhizal fungi (PP: 85 and 76%, FM1: 79 and 68%, FM2: 49 and 5%). The control in the long microcosm could therefore not be used for statistical comparisons. The shoot biomass of inoculated pigeon pea was significantly increased versus uninoculated controls (Fig. 2A). In pigeon pea, *R. fasciculatus* promoted the highest increase in shoot biomass with 263.2% in the short microcosm compared to the control (Fig. 2A). AMF inoculation also clearly improved nodulation of pigeon pea roots with rhizobia after visual assessment in both the short and long microosms (Table S1). For both finger millet plants in the short microcosm *R. fasciculatus* promoted growth significantly more than the other inoculants, however not more than the control. With the other two species, *R. irregularis* and *C. etunicatum*, total dry biomass of the finger millet plants was significantly decreased (*R. irregularis*: -46% finger millet 1 and -30% for finger millet 2 and C. etunicatum: -21% finger

millet 1 and -12% for finger millet 2). Interestingly, in the longer microcosm with *C. etunicatum* dry biomass (Fig. 2B) and dry panicle weight (Fig S1) of both finger millet plants was significantly higher than with the other species. No significant differences were found for 1000 seed weight in finger millet (Table S2). In general P content per plant was more improved by mycorrhization than biomass alone (Fig. 3). In the short microcosm in the more distant second finger millet when inoculated with *R. fasciculatus* the P content was significantly increased compared to the control (Fig. 3A). In the long microcosm *C. etunicatum* improved the P content greatly and significantly more than the other species (Fig. 3B). When compared to the control of the short microcosm it was an increase of more than threefold for the first finger millet and more than twofold for the second finger millet. P concentration in shoot and root was only significantly increased in pigeon pea (Fig S2 and S3).

All three AMF species colonized pigeon pea well (>85%). For the finger millets the colonization depended on the distance to the pigeon pea plant, with the more distant being less colonized. The colonization values had a large variance but in the short microcosm *R. fasciculatus* colonized the second finger millet the least and significantly colonized the first finger millet the least in the long microcosm. All first finger millets were well colonized by the other AMF species. *R. irregularis* appeared to colonize the most distant finger millet in the long microcosm the most, although this was not significant according to Tukey-HSD (Fig. 3D).

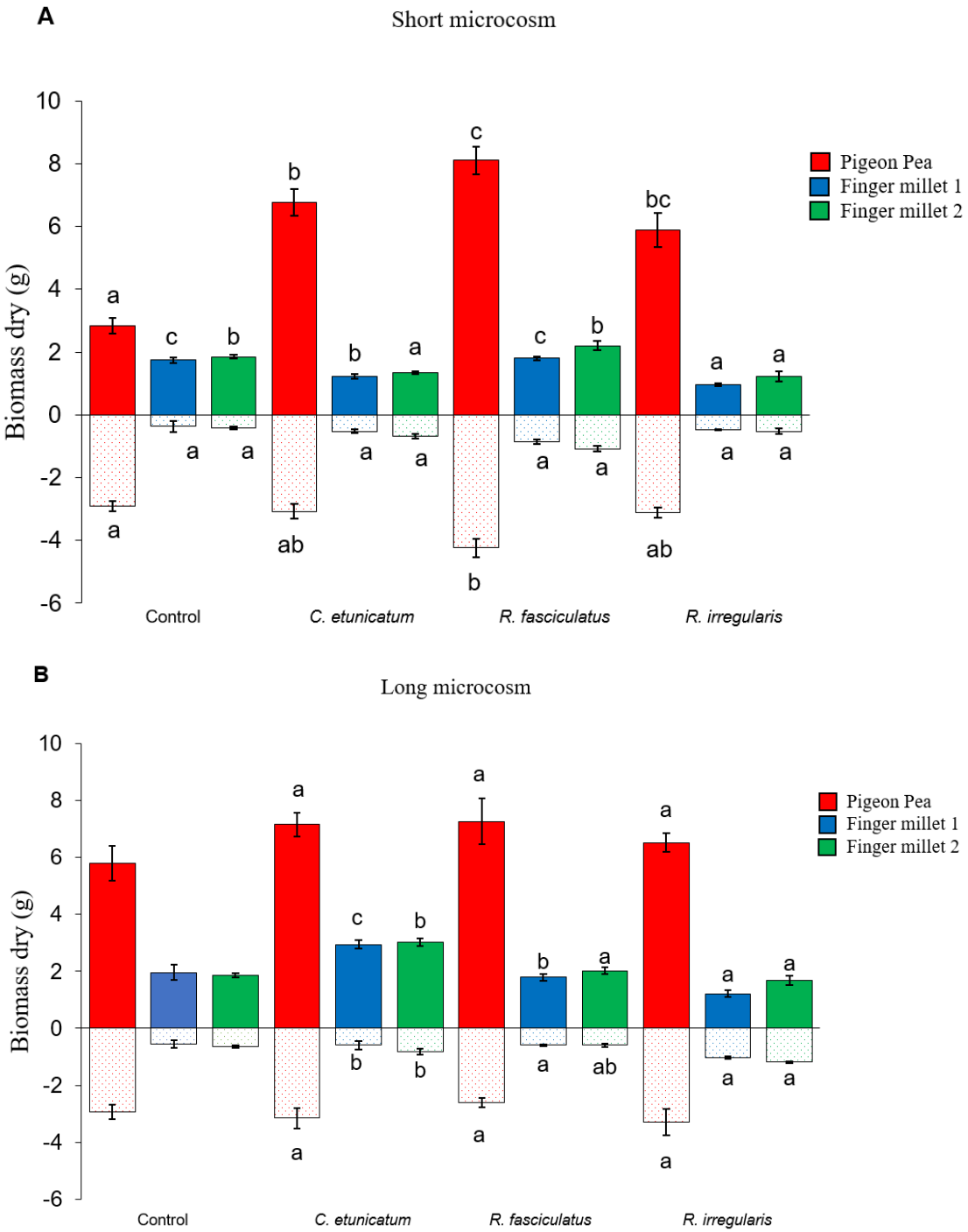

Figure 2: Biomass, shown separately for root and shoot, of pigeon pea and finger millet after inoculation of pigeon pea with different AMF. Red bars are results from pigeon pea, blue bars from finger millet 1 and green bars from finger millet 2. A depicts results from the short microcosms and B from the long microcosms. Tukey test was conducted within each pigeon pea, first finger millets and second finger millets, shoot and root respectively. The values represent the mean ± SE of four replicates. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. n=2 for control long microcosm (excluded from statistical analysis).

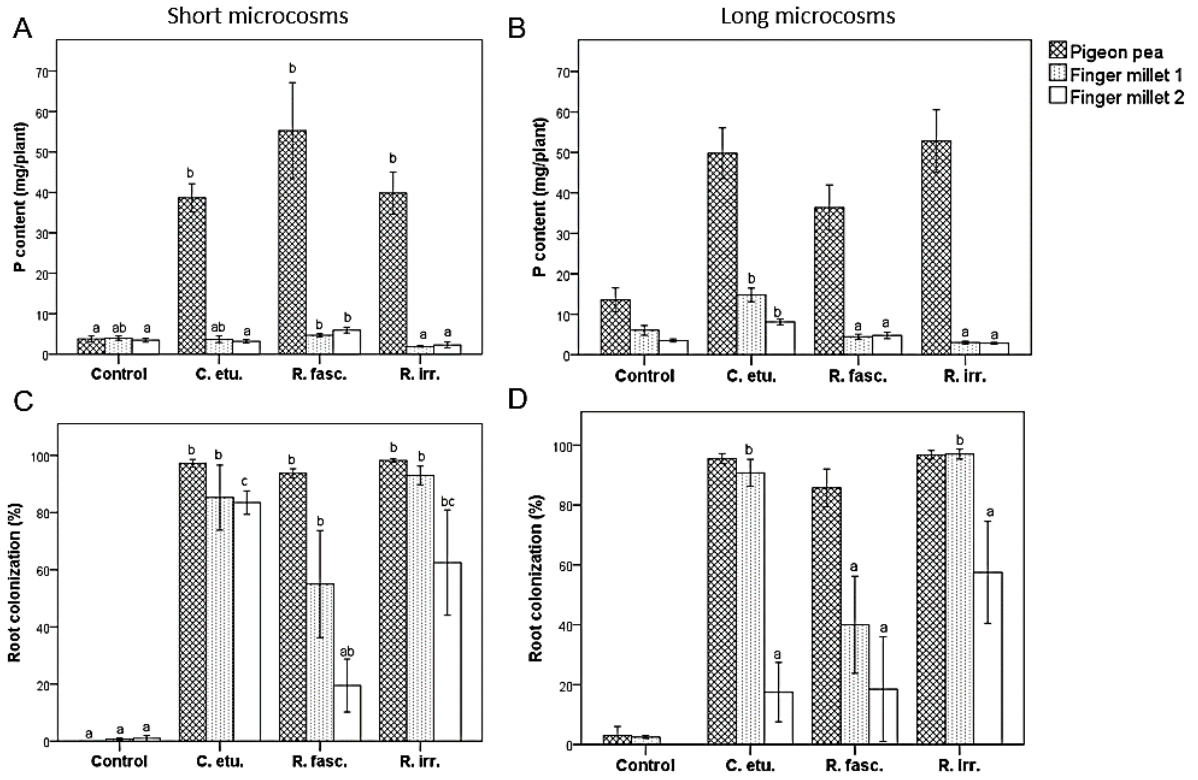

Figure 3: On the left side (A, C) findings in the short microcosms are displayed and on the right side (B, D) findings in the long microcosms. A and B show the phosphorus content of the plants. C and D display the colonization of the plants with AMF. Tukey test was conducted within each pigeon pea, first finger millets and second finger millets. The values represent the mean ± SE of four replicates. Columns labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. n=2 for control long system (excluded from statistical analysis).

Hyphal length densities (HLD) of all species was similar in the short microcosm showing that all AMF species covered the 5 cm hyphal compartment well. However *R. fasciculatus* which promoted growth of finger millet the most had its HLD slightly reduced after 14 days. In the long microcosm *C. etunicatum* was significantly better than the others and covered 10.2 cm at the sampling after 42 days with the highest HLD (15 cm g^{-1}). In the long microcosm it was clearer that *R. fasciculatus* had the least hyphal length densities at the two most distant sampling points and significantly different from *C. etunicatum* at the most distant sampling point. When grown without mesh the hyphal spread showed the same pattern and overall growth was not increased in the presence of roots (Fig. 4).

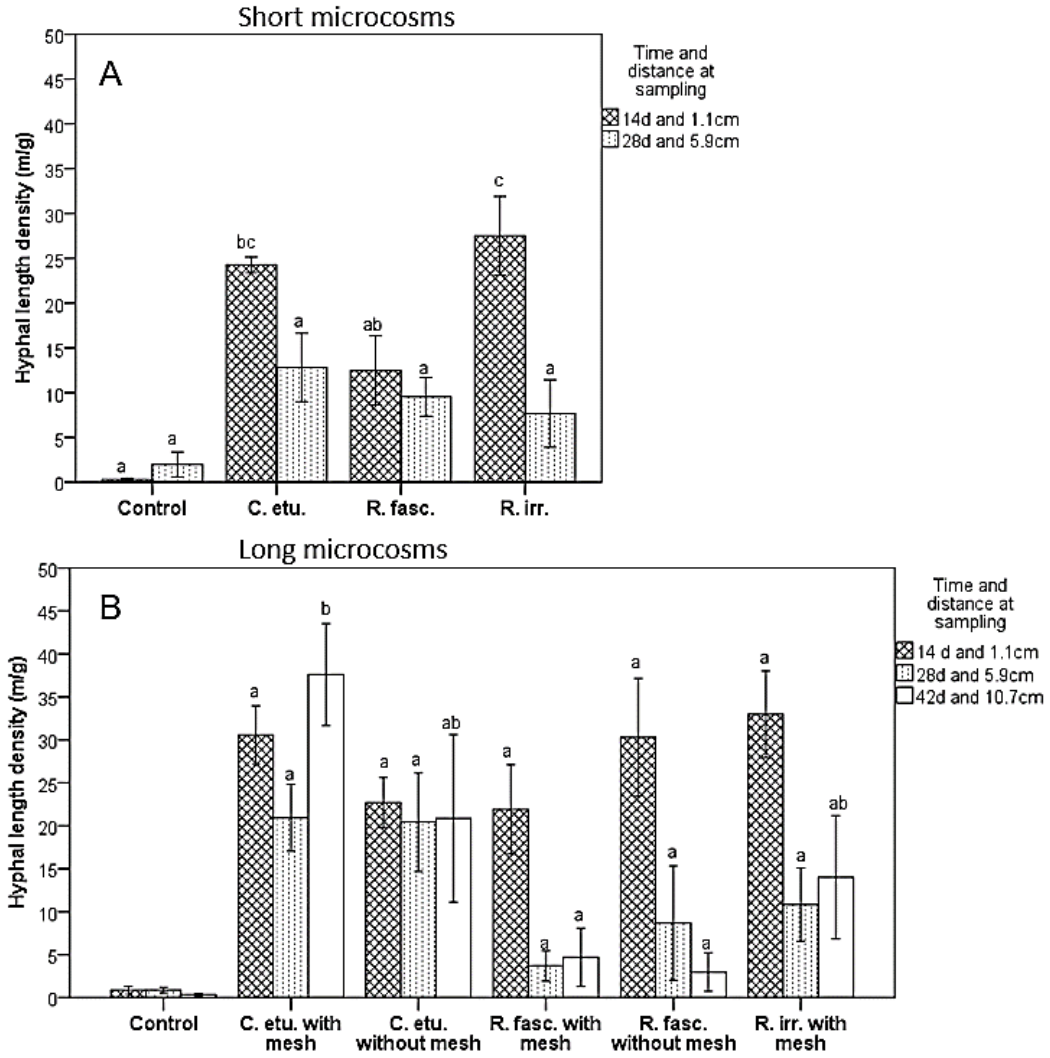

Figure 4: (A) depicts findings in the short microcosms and (B) in the long microcosms. Hyphae were extracted at different distances to the pigeon pea plant and time points. Tukey test was conducted within each sampling distance and time. The values represent the mean ± SE of four replicates. Columns labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. n=2 for control long system (excluded from statistical analysis).

Analysis of the nitrogen isotopes revealed that *C. etunicatum* and *R. irregularis* transported significant amount of ^{15}N label to the first finger millet in the short microcosm which was enriched up to 3.71% ^{15}N of total nitrogen content by *C. etunicatum* and 2.55% by *R. irregularis*. To a lesser extent also the second finger millet was fertilized through their hyphae which was enriched significantly to 1.87% by *C. etunicatum* only. ^{15}N was also transported to further distances in the long microcosm which was only significant for *C. etunicatum* and made up 3%. In both lengths of the microcosm *R. fasciculatus* did not transport any ^{15}N via their hyphae. Like the controls the level of ^{15}N was 0.4% which corresponds to the natural abundance of this nitrogen isotope and shows again that there was no transport of the tracer (Fig. 5).

Total N content in finger millet 1 and 2 in the short microcosm was never improved by AMF.

However with *R. irregularis* it was significantly decreased for finger millet 1 in the short microcosm (Table S3). Yet there were significant differences between the AMF species. In support to the biomass data in the short microcosm in both finger millet 1 and 2 *R. fasciculatus* improved total N content significantly more than *R. irregularis.* Although higher, the difference to *C. etunicatum* was not significant in both finger millet 1 and 2. Inoculation with *C. etunicatum* in the long microcosm in both finger millet 1 and 2 resulted in significantly higher values than when inoculated with *R. irregularis* or *R. fasciculatum* (Table S3).

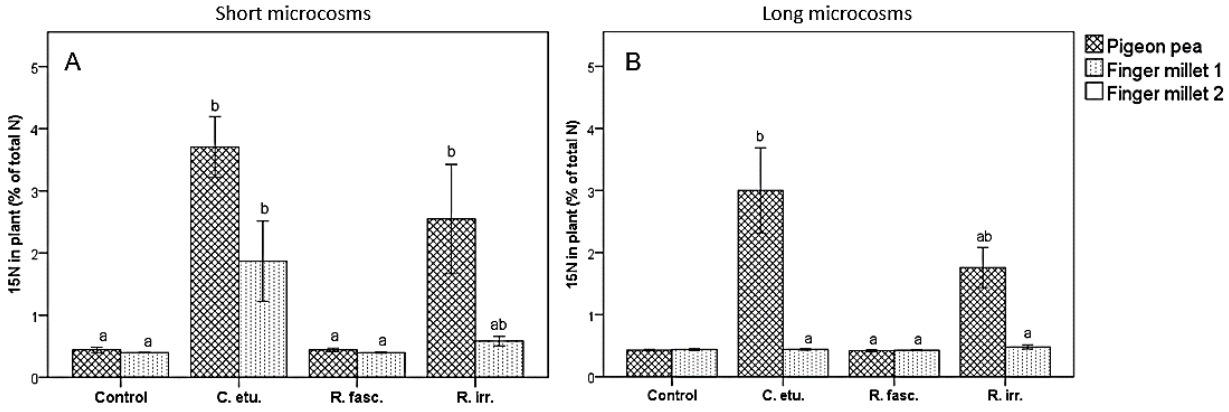

Figure 5: The left side (A) depicts findings in the short microcosms and the right side (B) in the long microcosms. Although *R. fasciculatus* was connected with pigeon pea, it clearly did not transport nitrogen as it was found with the other two AMF species. Tukey test was conducted within each first and second finger millets. The values represent the mean ± SE of four replicates. Columns labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. N=2 for control in long system (excluded from statistical analysis).

Estimating hyphal growth speed and the role of activated carbon

In the second experiment, with *C. etunicatum* in a "long microcosm" with one donor pigeon pea and only one receiver finger millet, hyphae covered the distance of 12 cm and colonized the neighbouring finger millet, confirming findings from the first part of our study. However two pigeon pea plants each after three and four weeks were not colonized and after five and 8 weeks had a very low colonization. Those were excluded from the analysis. Further treatments were excluded from the estimation of hyphal spread because of limited hyphal growth into the HC (6 microcosms in total). Their values were maximally one 6^{th} of the next largest replicate and did not exceed 3.9 m hyphae/g. These microcosm may have not been handled with enough care as small movements between the compartments can break the hyphae easily. Over the five time points hyphal length densities increased continuously until a certain plateau of about 40 m hyphae per g dry soil was reached (Fig 6).

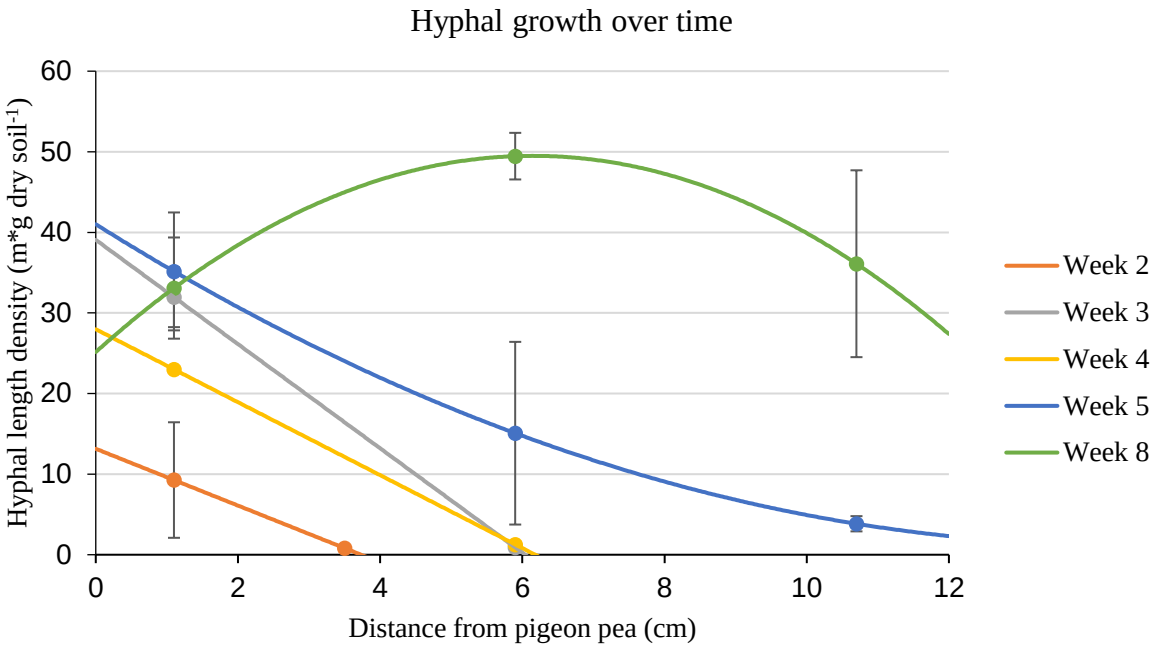

Figure 6: Hyphal length density at different distances from pigeon pea and 5 time points after HC was attached. Error bars depict SEM. For the samples of week 4 no SEM could be calculated. Week 2: n=5; Week 3: n=3; Week 4: n=2; Week 5: n=5; Week 8: n=4.

The full length of the HC was colonized between the 4th and 5th week after the HC was attached. By using data from all weeks on HLD without outliers a regression plane was fitted in R (for visualization see fig S5) with an R^2 of 0.54. The time of sampling was highly significant ($p=<0.0001$) and the distance to pigeon pea was moderately significant ($p=<0.05$) and the following formula was extracted for the regression plane:

$$HLD\ (m/g) = 3.75 + 4.73 * Week - 1076.53 * Distance\ (m)$$

Resolving the formula for HLD=0 and by setting Week to one 7th (1d) results in a distance of 4.1 mm which addresses the question at which distance does the HLD reach 0 in one day. This is the distance that hyphae grow in one day and a value for their hyphal spread and speed.

After 8 weeks pigeon pea was well colonized by AMF hyphae and also many arbuscules could be found (Fig S6), either with AC or not. This is reflected also in the growth promotion (Fig 7). The roots were also well nodulated, and first nodules were already observed at the first harvest (Fig S10). For finger millet however, the colonization level by AMF and rate of arbuscule formation (Fig S6) was low, but the growth was significantly increased in the AMF treatments (Fig 7). For the following comparisons with the control without AMF inoculation, it is assumed that the layer of AC had no influence itself on the growth of the plants. This is further discussed

later on in the discussion section. Yet the finger millets in the microcosms with AC were significantly smaller than the AMF treatments and remained the same as the control (Fig 7). The same trend was found in the shoot length of both plants (Fig S8). The results, however, were less clear, showing a slight height difference for AMF treated finger millet already after three weeks and only after 6 weeks in finger millet were the controls significantly ($p<0.05$) shorter than the AMF treated ones. The P content in AMF treated pigeon peas was significantly larger than the controls and the ones with AC were again significantly larger than AMF treated plants. For finger millets no significant effect could be found (Fig 8 for total P content per plant, and Fig S7 for the concentration of P in shoots and roots, respectively).

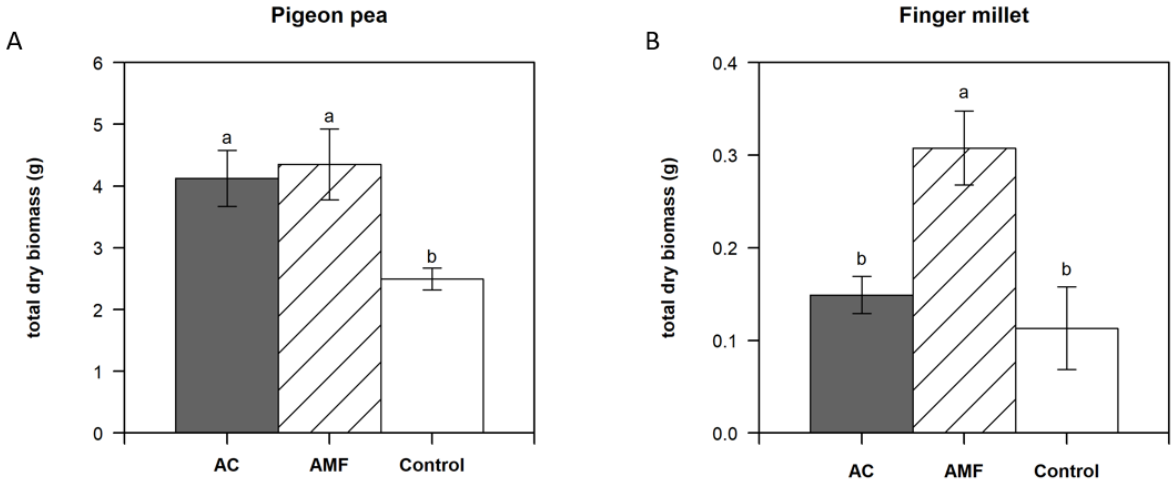

Figure 7: Dry biomass of (A) pigeon pea and (B) finger millet with AMF inoculation in the compartment of pigeon pea and without, and with AMF inoculation in the compartment of pigeon pea but with a layer of AC in the middle of HC.

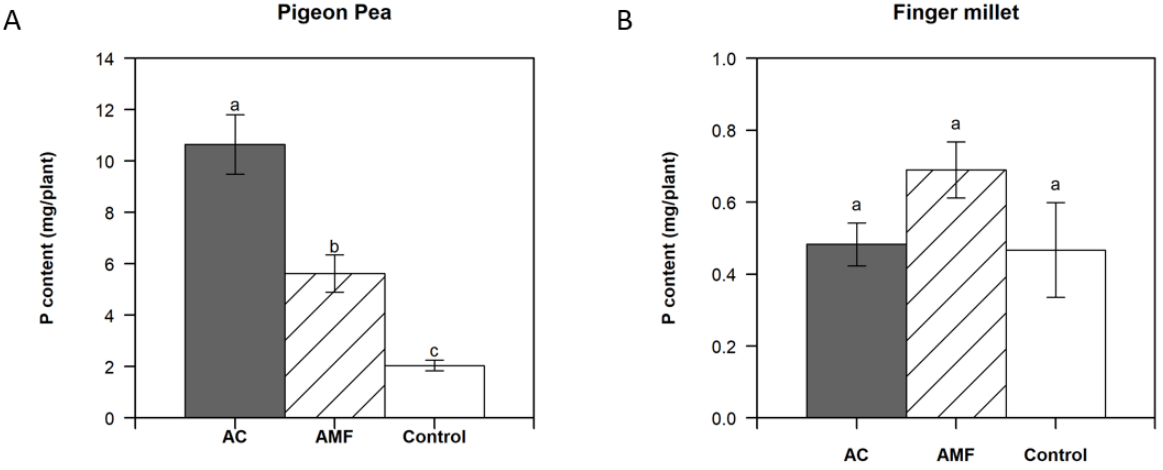

Figure 8: Phosphorus content of (A) pigeon pea and (B) finger millet with AMF inoculation in the compartment of pigeon pea and without, and with AMF inoculation in the compartment of pigeon pea but with a layer of AC in the middle of HC.

Discussion

We found that AMF hyphae can spread readily through root free soil bridges from the roots of pigeon pea to the roots of finger millet, covering distances of up to 35 cm in 20 weeks, and have growth promoting effects there. This is the first time that such a long microcosm was used to simulate intercropping conditions and the spread of mycorrhizal hyphae between the rows.

Hyphal spread was measured by several studies (Jakobsen et al. 1992; Harinikumar and Bagyaraj 1995; Jansa et al. 2003), but never between two different plant species. Our study not only assessed the spread of hyphae, but also the compatibility of different AMF species with pigeon pea and finger millet.

Most favourable AMF species

Pigeon pea benefitted significantly from mycorrhization by all three tested AMF species, both with regard to biomass and P content, in the experiment with short microcosms. In the short microcosm the biomass of pigeon pea almost tripled when inoculated with *R. fasciculatus*. In the long microcosms this could not be confirmed because two of four controls were colonized by AMF. Such a growth promotion is not unusual in laboratory experiments and had been found also in other studies (Saharan et al., in preparation). Although all AMF species reached and colonized the most distant finger millet after 5 months, their HLD at three sample distances and sample times differed. Our sampling strategy assumed that the hyphae would regrow into the freshly supplied soil and/or hyphae would continue growing from outside the removed soil core. In this experiment we cannot exclude that differences in the HLD between the three AMF from the second sampling onward measured also their ability to recover from the previous sampling. Different hyphal growth strategies were found for the different species and include the unknown component of the recovery from sampling. *R. fasciculatus* has been shown to be the best isolate out of three local AMF strains and one American strain for growth promotion in finger millet (Govinda Rao et al. 1983). But here *R. fasciculatus* promoted growth of finger millet only in the short microcosms. In the long microcosms it only showed a low root colonization and low HLD at the two largest distances and later sampling time. Instead *C. etunicatum* only promoted the growth of finger millet in the long microcosms. The finger millet plants had a greater biomass than in the short microcosms inoculated with *R. fasciculatus*. *R. irregularis* had no effect on growth of finger millet despite its over all distances and sampling times higher densities of hyphae. Thus *R. irregularis* forages for nutrients but seems invest little of it in the growth of finger millet. Data on P content per plant reflect the differences found in biomass yet the differences are larger. Different strategies in *Rhizophagus irregularis* (*Glomus*

intraradices), *Claroideoglomus etunicatum* (formerly *Glomus etunicatum*) and *Gigaspora gigantea* were also identified by Hart and Reader in a root observation chamber without a HC (Hart and Reader 2005). *R. irregularis* and *C. etunicatum* with extensive root colonization produced also many external structures like runner hyphae, absorptive hyphae and hyphal bridges. *R. irregularis* showed an earlier (3 weeks) colonization than *C. etunicatum*, but it also reached a plateau of external structures earlier. Although not significant in our results there is a similar trend with less HLD values at larger and later sampling than for *C. etunicatum.*

Hyphal spread

If the hyphal spread would be calculated as if the hyphae had reached the sampled hole on the sampling day for *C. etunicatum*, the fastest species, this translates to a speed of 2.43 mm d^{-1}. Nevertheless HLD indicate that on that day hyphae have already grown further. In our second experiment with destructive sampling this speed was in fact higher with 4.1 mm per day.

To our knowledge this result is the fastest hyphal spread of AMF recorded. With *Trifolium subterraneum* Jakobsen et al. (1992) found the maximum hyphal growth of 3.1 mm d^{-1} with *Acaulospora laevis* at a similar distance inside a hyphal compartment at 7 and 11 cm, however without a plant compartment behind. Also much slower rates have been detected e.g. 0.6 mm d^{-1} in *Glomus fasciculatum* and *Festuca rubra*, however estimated via the root colonization (Warner and Mosse 1982). Hyphal growth of course depends on soil parameters. Temperature and moisture have been found important factors for the growth and dieback of hyphae (Hernandez and Allen 2013). Comparable rates were measured in ectomycorrhizal fungi (Finlay and Read 1986). In the microcosm without separating meshes and unlimited root growth we observed roots of pigeon pea throughout the microcosms at harvest time, but could not find significant differences in HLD in all distances and at all times to the ones with HCs. This is surprising because hyphae would be expected to have a higher density when in closer contact with roots and when roots and hyphae are exploring the soil together with AMF producing a higher quantity of absorptive hyphae than runner hyphae which colonize new soil patches (Bago et al. 1998). Roots have faster growth rates than fungi (Bengough and Mullins 1991; Bengough et al. 1997) which should also enable AMF to explore the soil faster.

Nitrogen transport

Furthermore in the first experiment we could show that nitrogen can be transported across distances up to 12 cm by AMF. There was no diffusion of 15N through the hyphal compartment to finger millet as the control had a very small δ15 value close to the one in the atmosphere.

The idea that nitrogen can benefit an intercropped cereal is not new. In our case we cannot distinguish whether 15N was taken up by the legume and then transferred to AMF or whether 15N was taken up directly by AMF. Some N assimilated by the finger millet may have been derived from the intercropped pigeon pea; such transfer from non-legume to legume has been shown by several studies (Giller et al. 1991; Jensen 1996; Chu et al. 2004). N is excreted by legumes via root exudates or dead nodules which is then either reabsorbed by the legumes or taken up by soil microorganisms or another plant (Ledgard and Steele 1992). An increased N transfer from legumes by AMF inoculation has been found (Johansen and Jensen 1996; Meng et al. 2015), and has been suggested to follow a source sink relationship between N donor and recipient plants (Smith and Smith 1990; Bethlenfalvay et al. 1991; Jalonen et al. 2009). The other part of the transferred nitrogen has been taken up by AMF hyphae directly representing 15N like an N fertilizer. Unlabelled ammonium nitrate was supplied to pigeon pea only and the increased total N values of finger millet in AMF inoculated treatments is another indicator for the transport of elemental N taken up from the soil. Ammonium seems to be the form of nitrogen that is most transported by AMF hyphae to the root versus nitrate which is taken up but less readily transferred; Tanaka et al. (2005) found a 10 times higher amount transported to the root by *Glomus aggregatum* of ammonium versus nitrate. Three ammonium transporters are known in *R. irregularis* with one having been recently discovered (Calabrese et al., 2016).
The lack of 15N transport by *R. fasciculatus* might be caused by a different set of ammonium transporters. Another possible reason is a differing compatibility with finger millet, which does not involve the transport of nitrogen but only e.g. phosphorus. Such difference in nutrient transport where either P or N is increasingly transported by one AMF species were also found by Walder et al. (2012) for *Glomus intraradices* and *Glomus mosseae*. Total N transfer depends also on the AMF species. Large differences depending on the fungal isolate were found in the study by Mårtensson et al. (1998) N obtained by chicory from pea ranged from three to fifty percent of total N in a root separated microcosm.

Suitability of different AMF species as inoculants for different planting densities

We characterised three AMF species in pigeon pea finger millet intercropping system, yet the final growth promotion of finger millet by AMF was variable with no growth promotion in the short microcosms and significant increases in the long microcosms. Finger millet 1 was connected with AMF hyphae of *C. etunicatum* after about four to five weeks of age and probably also colonized soon after. It is possible that in the short microcosm the hyphae transported nutrients mostly to pigeon pea, having little growth promotion on finger millets but

that this effect disappeared in the longer microcosm. The pigeon pea plants were one month older and had also in the end of the experiment much more biomass. AMF have been shown to favour plants which are older and have a higher rate of photosynthesis (van der Heijden and Horton 2009). In the field, the density and distance between plants has been shown to be crucial for the success of an intercropping system (Dhima et al. 2013) and also for pigeon pea- finger millet intercropping systems (Padhi et al. 2010).

Allelopathic substances travel along AMF hyphae

One other reason may be "allelopathy", i.e. root exudates of one plant inhibiting growth of another (Reigosa et al. 2006). Indeed, it has been found that AMF can transport allelopathic substances such as juglone (Achatz and Rillig 2014). Pigeon pea is known to contain substances with an allelopathic potential (Hepperly et al. 1992). Such allelopathic substances could be another reason for the lower growth promotion in the short microcosms.

Activated carbon and hyphal growth

The growth of finger millet was clearly reduced when there was a layer of AC in the HC which however assumes that the layer of AC had no effect itself on the growth of the two plants. Two replicates showed a colonization by AMF. We would have expected no colonization to support our hypothesis that signalling molecules like strigolactones from finger millet are blocked by AC and preventing AMF hyphae to grow past the layer of AC. The results are too variable to conclude anything by certain, yet the data suggest that the layer of AC has slowed down hyphal growth. This may be due to a hole in the AC layer or indeed indicate that hyphae explore the soil also without knowing about surrounding plants.

Interestingly the P content was greatly increased in pigeon pea when there was AC in addition to AMF. It is indeed possible that AC contained some P which was transported by the hyphae and which we then found in the pigeon pea. This indicates the need for a control without AMF inoculation and a layer of AC to learn about the function of the AC and whether nutrients dissolve into the surrounding soil and reach the plants.

Conclusions

We conclude that the row distance between the crops and the choice of AMF species play a crucial role for the application of AMF as biofertilizers in the pigeon pea-finger millet intercropping system. It is important to select AMF species not only for their compatibility with both plant species but to also consider the planting scheme and other interactions with the

environment. For example in real farming situations, the finger millets are exposed to naturally occurring AMF species. The hyphal growth from the inoculated pigeon pea is fast and would connect with the first rows of finger millet and would have to compete there with the natural AMF species. So if an AMF species with a fast hyphal growth is selected for pigeon pea, like *R. irregularis* in our study, it would connect easily with the first rows of finger millet, but needs to compete there and also be compatible with finger millet. It may instead through the competition with other AMF species invest more in this competition and not in the growth of the finger millet. The three AMF species that we tested had some influence on finger millet but at this stage, we cannot recommend any of them for field application. The novel design of our microcosms for the study of intercropping in the greenhouse however proved to simulate field conditions well and let us study the complex interactions in intercropping in great detail.

Acknowledgement

This work was supported by the “IDP Bridges program” (LS), the Indo-Swiss Collaboration in Biotechnology (ISCB) (TB, PM, MN) and “Plant Fellows” (KS). We also acknowledge the isotope analysis by Dr. Victor Evrard which was conducted in the group of Sustainable Land Use of Professor A. Kahmen.

Supplement

Table S1: Visual rating of nodulation on a scale from 1-5 with 1 meaning no nodulation and 5 the most intense nodulation of pigeon pea roots in the short and long microcosm after inoculation with different AMF species. N=2 for control long microcosm

Microcosm length	Treatment	Rating of nodulation / plant*
Short	Control	1.66 ± 0.29
Short	C. etunicatum	3.75 ± 0.25
Short	R. fasciculatus	2.75 ± 0.25
Short	R. irregularis	2.75 ± 0.25
Long	Control	1.5 ± 0.29
Long	C. etunicatum	3.75 ± 0.25
Long	R. fasciculatus	2.75 ± 0.48
Long	R. irregularis	3.5 ± 0.29

* Means ± SE

Table S2: 1000 seed weight in the short and long microcosm after inoculation with different AMF species. Finger millet 1 is the plant closest to pigeon pea and finger millet 2 the more distant plant. Letters after the values show a significant difference (P>0.05) within each group according to Tukey test. N=2 for control long microcosm

Microcosm length	Treatment	Finger millet 1 1000 seed weight (g)*	Finger millet 2 1000 seed weight (g)*
Short	Control	3.49 ± 0.22 [a]	2.92 ± 0.05 [a]
Short	*C. etunicatum*	3.28 ± 0.24 [a]	3.46 ± 0.28 [a]
Short	*R. fasciculatus*	3.21 ± 0.18 [a]	3.18 ± 0.16 [a]
Short	*R. irregularis*	3.43 ± 0.18 [a]	2.95 ± 0.12 [a]
Long	Control	3.15 ± 0.24	2.85 ± 0.20
Long	*C. etunicatum*	3.41 ± 0.18 [a]	3.23 ± 0.04 [a]
Long	*R. fasciculatus*	3.44 ± 0.24 [a]	3.16 ± 0.14 [a]
Long	*R. irregularis*	3.25± 0.09 [a]	3.21± 0.25 [a]

* Means ± SE; Treatments labeled with the same letter after the value are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA

Table S3: Total N content of finger millet 1 and 2 in the short and long microcosm after inoculation with different AMF species. Finger millet 1 is the plant closest to pigeon pea and finger millet 2 the more distant plant. Letters after the values show a significant difference (P>0.05) within each group according to Tukey test. N=2 for control long microcosm

Microcosm length	Treatment	Finger millet 1 Total N content (mg)*	Finger millet 2 Total N content (mg)*
Short	Control	9.7 ± 0.7 [b]	9.5 ± 0.8 [ab]
Short	*C. etunicatum*	8.7 ± 0.2 [ab]	9.1 ± 0.3 [ab]
Short	*R. fasciculatus*	9.8 ± 0.3 [b]	10.8 ± 0.6 [b]
Short	*R. irregularis*	6.6 ± 0.4 [a]	7.2 ± 0.9 [a]
Long	Control	11.8 ± 0.5	8.8 ± 0.5
Long	*C. etunicatum*	18.9 ± 1.1 [b]	15.8 ± 0.7 [b]
Long	*R. fasciculatus*	9.2 ± 0.4 [a]	10.2 ± 0.6 [a]
Long	*R. irregularis*	8.2 ± 0.9 [a]	8.8 ± 0.5 [a]

* Means ± SE; Treatments labeled with the same letter after the value are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA

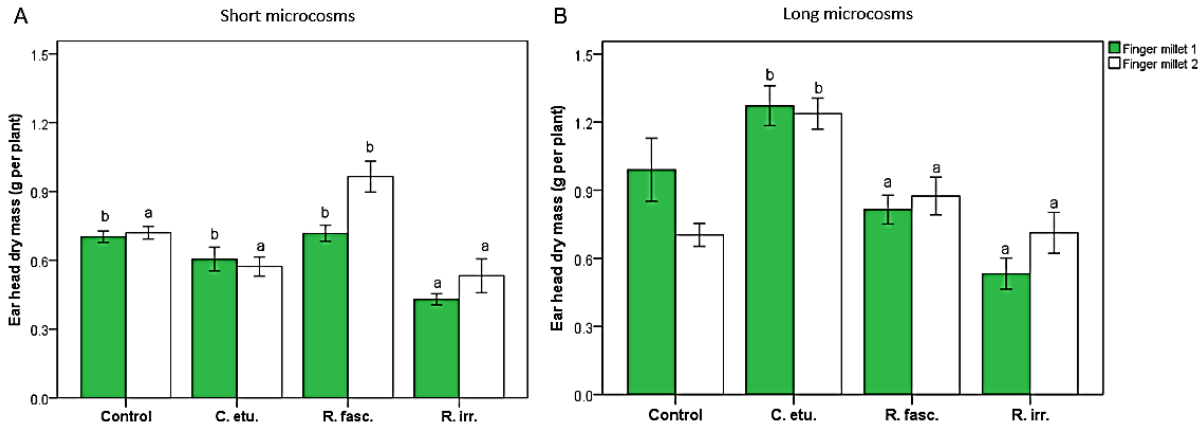

Figure S1: The left side (A) shows the results from the short system and the right side (B) from the long system. Finger millet 1 is the plant closest to pigeon pea and finger millet 2 the more distant plant. Ear heads were dried separately and confirm the findings of the other data on biomass: The distance between the plants favors different AMF species. Tukey test was conducted within each first and second finger millets. The values represent the mean ± SE of four replicates. Columns labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. N=2 for control in long system

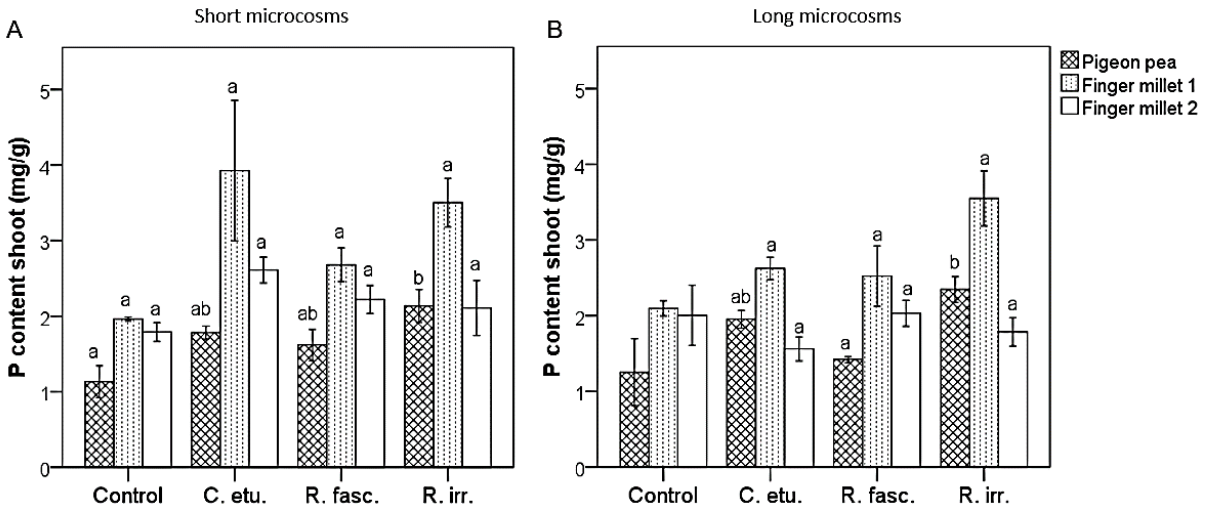

Figure S2: The phosphorus content per g dry biomass depending on mycorrhizal inoculum is shown. On the left side (A) findings in the short system are displayed and in the right side (B) findings in the long system. Tukey test was conducted within each pigeon pea, first finger millets and second finger millets. The values represent the mean ± SE of four replicates. Columns labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. n=2 for control long system (excluded from statistical analysis).

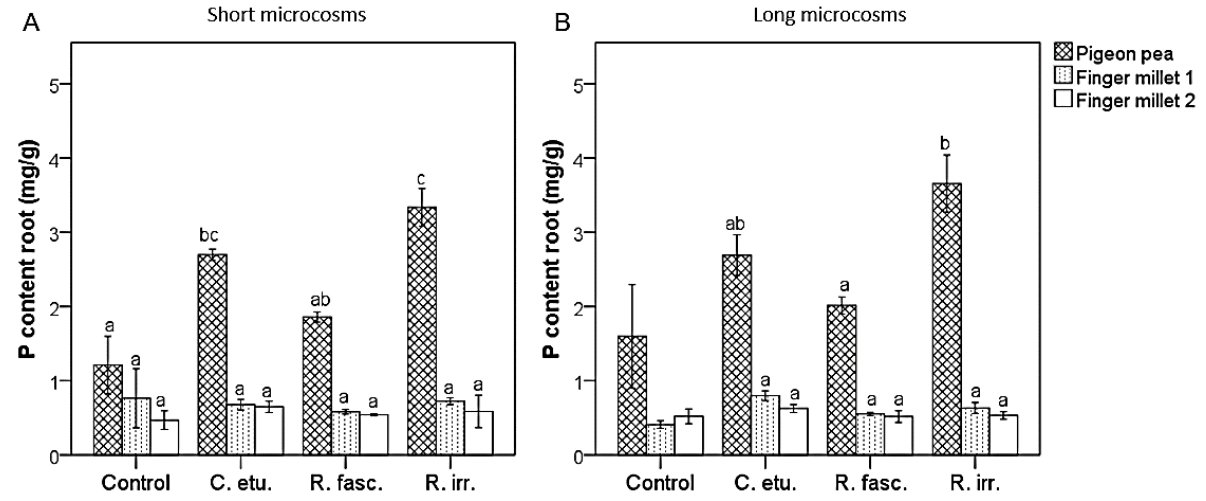

Figure S3: The phosphorus content per g dry biomass depending on mycorrhizal inoculum is shown. On the left side (A) findings in the short system are displayed and in the right side (B) findings in the long system. Tukey test was conducted within each pigeon pea, first finger millets and second finger millets. The values represent the mean ± SE of four replicates. Columns labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA. n=2 for control long system (excluded from statistical analysis).

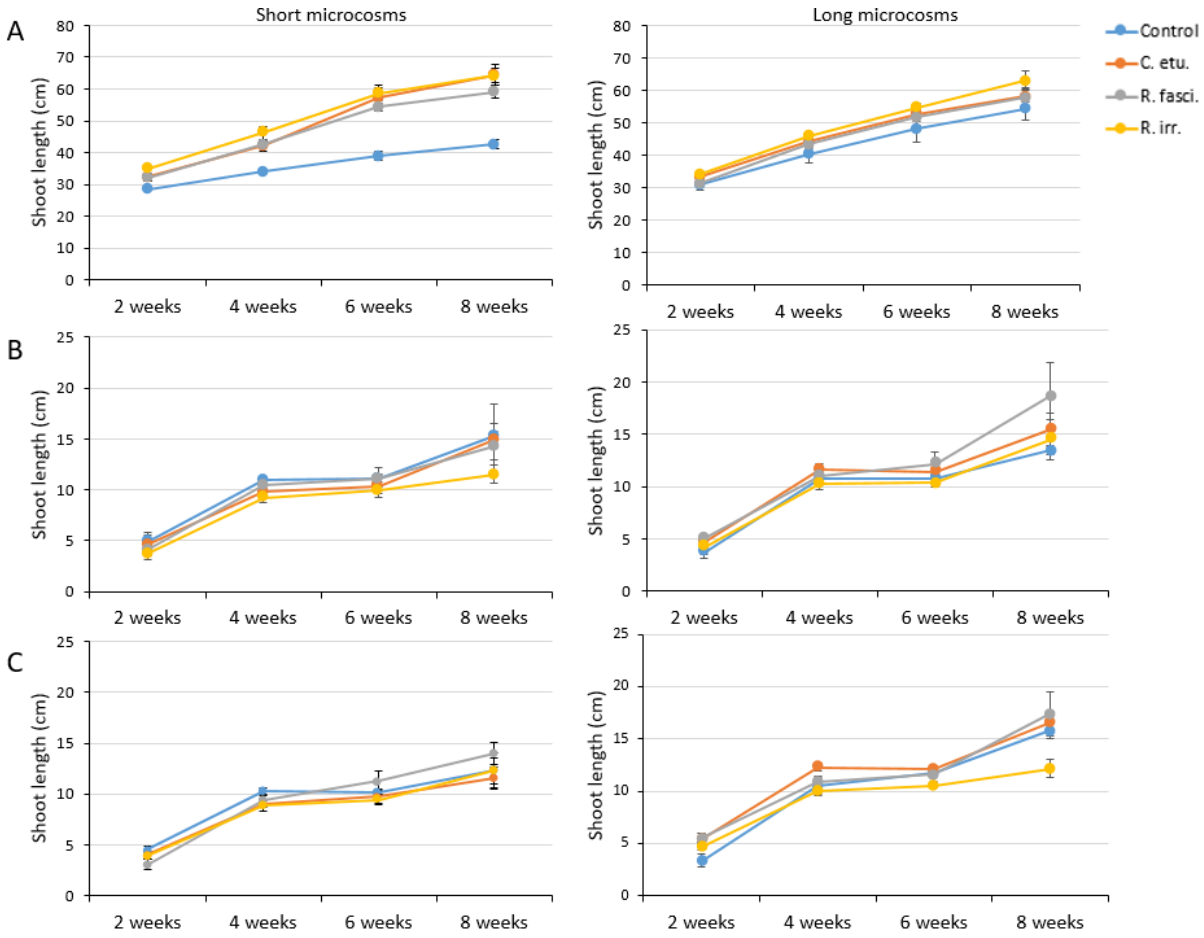

Figure S4: Shoot length of the plants over time and time after systems were joined together. On the left side findings in the short system are displayed and in the right side findings in the long system. A shows growth of pigeon pea, B of finger millet 1 and C of finger millet 2. The values represent the mean ± SE of four replicates. n=2 for control long system.

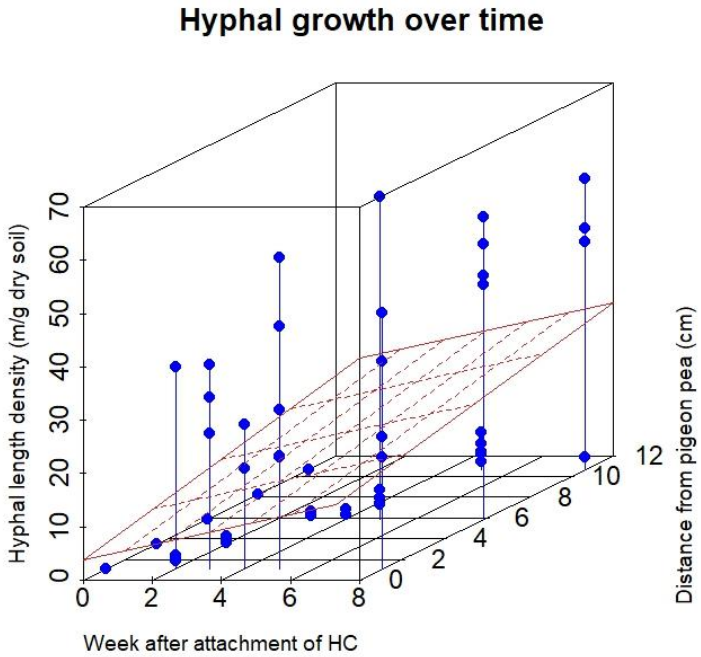

Figure S5: Hyphal growth over time without outliers. Blue lines join the measurements within one replicate for a better overview. The red layer is the regression plane which was was fittted through all data with an R^2 of 0.54.

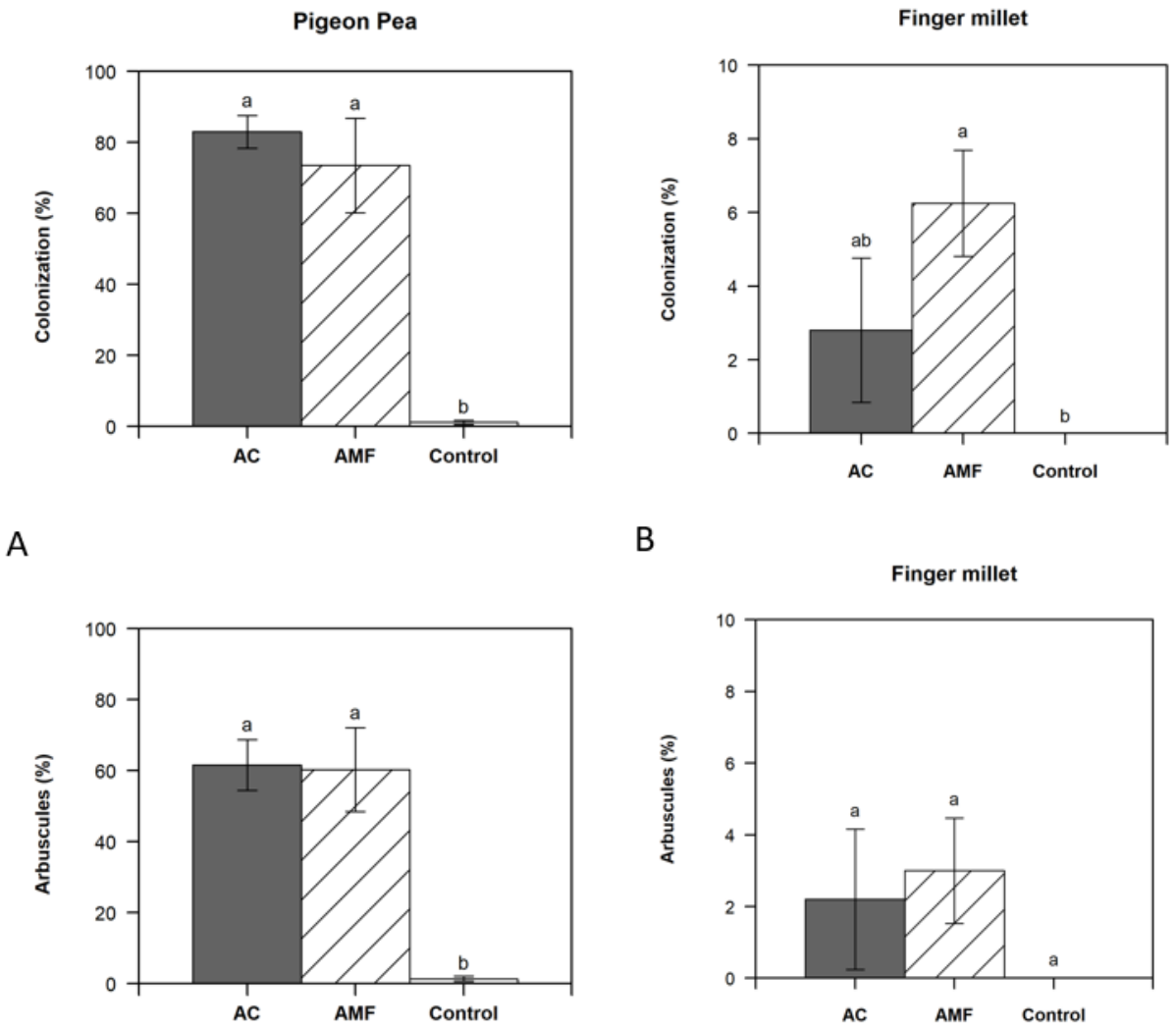

Figure S6: Relative root colonization and arbuscules presence in (A) pigeon pea and (B) finger millet with AMF inoculation in the compartment of pigeon pea and without, and with AMF inoculation in the compartment of pigeon pea but with a layer of AC in the middle of HC.

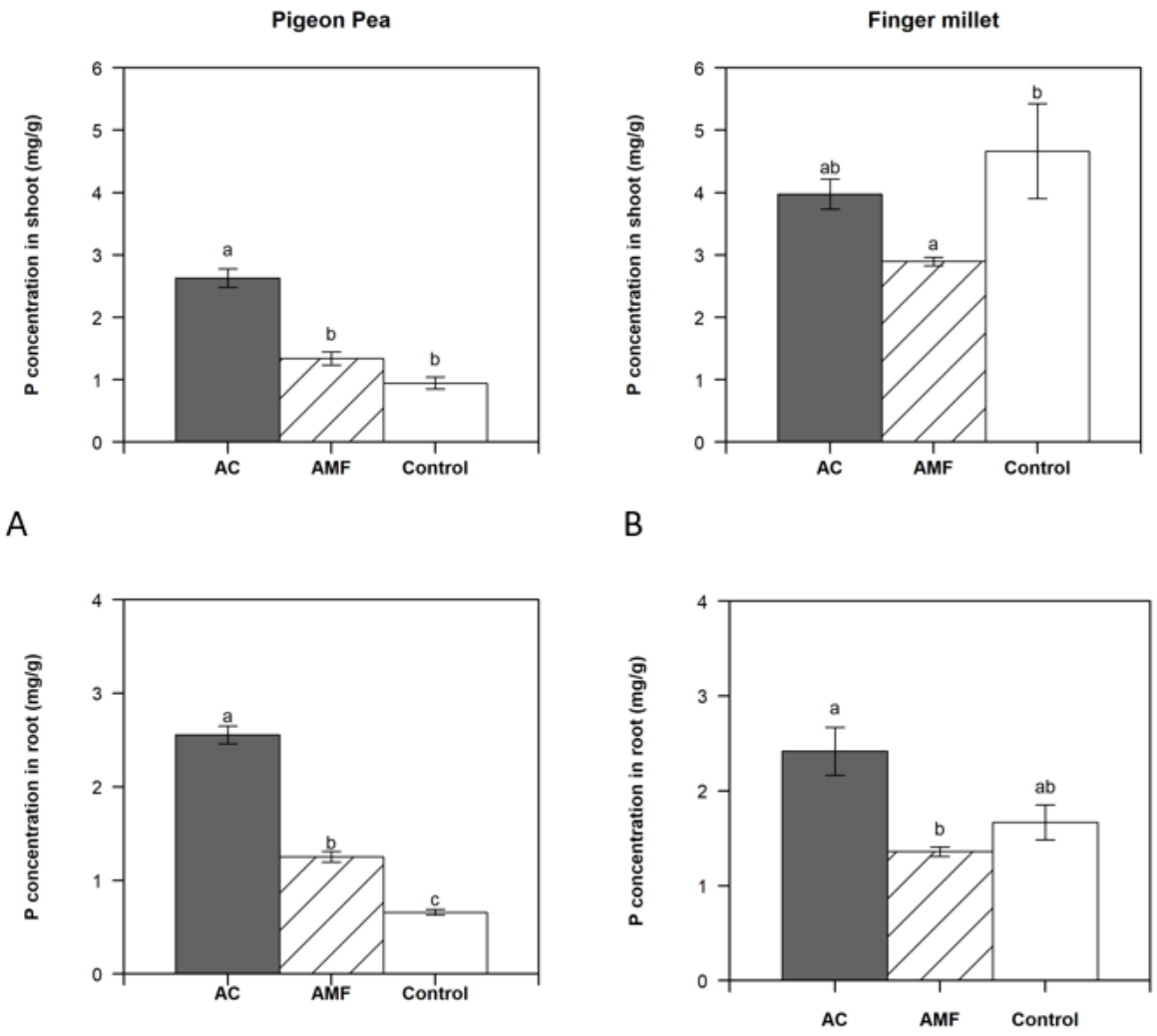

Figure S7: Concentration of phosphorus in root and shoot of (A) pigeon pea and (B) finger millet with AMF inoculation in the compartment of pigeon pea and without, and with AMF inoculation in the compartment of pigeon pea but with a layer of AC in the middle of HC.

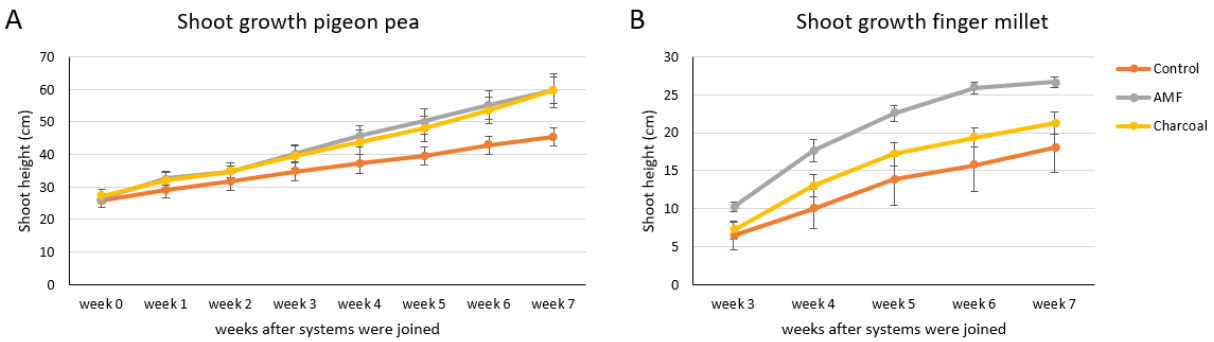

Figure S8: Shoot growth over time of (A) pigeon pea and (B) finger millet with AMF inoculation in the compartment of pigeon pea and without, and with AMF inoculation in the compartment of pigeon pea but with a layer of AC in the middle of HC.

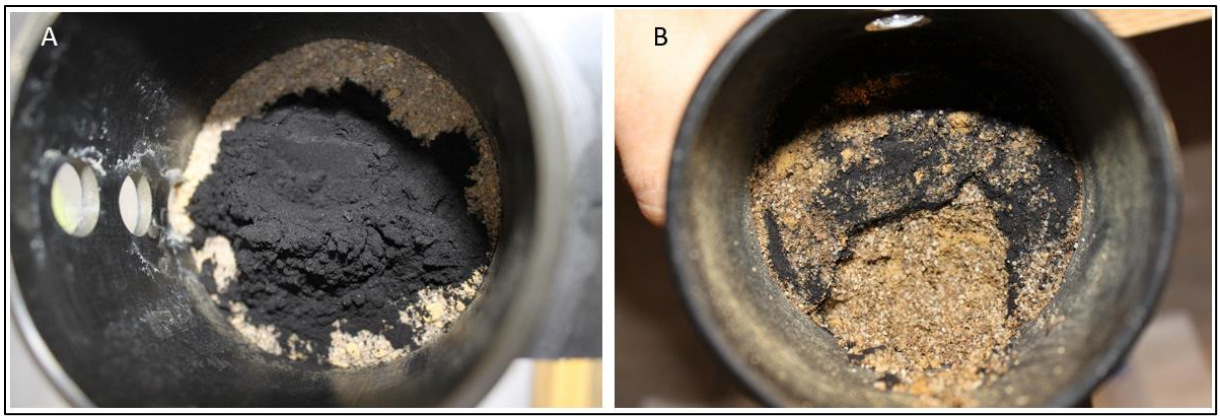

Figure S9: Activated carbon layer in the hyphal compartment while filling HC with substrate (A) and after 8 weeks at time of harvest (B).

Figure S10: Nodulation of pigeon pea at the first harvest (age 5 weeks). Finger millets plants are one week old.

The following study has been conducted by me, Lukas Schütz, and I am the main author.

The capability of pigeon pea (*Cajanus cajan*) to solubilize immobile forms of P and the ability of AMF to help plants for their P nutrition has no benefit for finger millet (*Eleusine coracana*)

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Abstract

Intercropping is practised in agriculture since long times as a risk aversion strategy, to increase yields and increasingly for environmental reasons. The mixing of two plant species can be positive for a better resource partitioning by for example the exploration of different soil layers for nutrients. But one crop can also facilitate the uptake of nutrients for the other crop by the excretion of root exudates.

We were interested in the potential benefit of intercropping of pigeon pea, a legume, and finger millet, a C4 plant, a common combination in South Indian agriculture, and in the role of the common mycorrhizal network connecting the two crops. Pigeon pea is known for its capability to solubilize iron-bound phosphorus. The focus of this study was to test whether this capability also benefits finger millet in terms of P nutrition as a model for P inaccessible to finger millet. Since AMF are well known for their role in P nutrition we also studied the role of AMF in this interaction and whether hyphae can form a connection between the two plants which can transport root exudates or P.

It was found that pigeon pea benefitted strongly from both AMF and $FePO_4$ addition with respect to biomass and P content. The biomass and P uptake of finger millet however decreased when grown with pigeon pea and no growth promotion by AMF was observed although the roots were only colonized when grown with pigeon pea. Furthermore it was found that finger millet cannot solubilize $FePO_4$ on its own and that mycorrhization did not affect the root diameter or length of both plants. We conclude that the capability of pigeon pea to solubilize immobile forms of P was of no benefit to finger millet.

Keywords: Biofertilizer, Intercropping, Pigeon pea (*Cajanus cajan*), Finger millet (*Eleusine coracana*), Iron Phosphate, Arbuscular mycorrhizal fungi (AMF), Compartment

Introduction

Soils especially in the tropics are low in soil available phosphorus and high in metals like iron, aluminium and calcium which immobilize phosphorus. In fact the main part is only present in immobile form. Fertilization has been the only way to increase P availability. Phosphate fertilizers are mined from a few rock phosphate deposits in the world. The peak of extraction of phosphate is expected to happen in the 2030s (Cordell et al. 2009) and agriculture is the main consumer of phosphorus in the form of mineral fertilizers. But the P recovery of these fertilizers is usually rather low (Syers et al. 2008). It is thus of paramount importance to study agricultural systems which can mobilize immobile forms of phosphorus.

Mixed cropping and intercropping describe the planting of two or more different crop species at the same time or slightly shifted which is called relay-cropping. This practice increases the diversity in agriculture and can at the same time increase yields when compared to mono-cropping of each crop alone on the same area of land. Such yield increase is called overyielding. Interspecific plant interactions change the use of resources (Vandermeer 1989; Zhang and Li 2003). Typically, successful plant combinations either use resources complementarily by making better use of the existing resource or one facilitates the uptake of a nutrient for the other by increasing the pool of nutrients. Complementary use of resources can happen through various mechanisms like complementary root architecture which enables the plant combination to make the best use of space for taking up nutrients and water from all soil layers. Facilitation can happen in all combinations with legumes when some of the fixed nitrogen by rhizobial symbiosis is made available to the other crop (Hauggaard-Nielsen and Jensen 2005; Li et al. 2007). But the facilitation between the two crops also depends on abiotic factors like nutrient availability, soil type or climate (Wang et al. 2007). Mixed cropping is often discussed to be especially suited for marginal lands (Qiao et al. 2015). Mixed cropping can also act as a buffer against extreme events when one crop is more resilient than the other which stabilizes yields over time.

Mixed cropping has also been shown favourable for the P nutrition. Studies have found that plants like faba bean or peanuts have root exudates which solubilize immobile forms of P which in turn were able to facilitate the access to P for other plants. Xia et al. (2013) found higher shoot P contents through intercropping maize/turnip, maize/faba bean, maize/chickpea and maize/soybean by 44.6%, 30.7%, 39.1%, and 28.6%, compared to sole maize crop, while yields were also improved. Overyielding in other maize/faba bean intercrop studies was also attributed to P facilitation in field and pot experiments (Li et al. 2003, 2010). Two mechanism are involved in the increased uptake of phosphorus which are not easy to distinguish experimentally

(Hinsinger et al. 2011). Complementarity happens by the use of different P pools, chemical or from different soil depth, by the different plants of the system. Facilitation describes the facilitated uptake of phosphorus by the other crop through for example the excretion of root exudates. Exuded protons/hydroxyls and carboxylates solubilize inorganic P, while root-borne phosphatases hydrolyse organic P (Hinsinger 2001; Vance et al. 2003). Through the excretion of piscidic acid, pigeon pea has a strong ability to solubilize immobile forms of P like calcium phosphate, aluminium phosphate or iron phosphate (Ae et al. 1990), which are present in highly eroded soils, and could thus facilitate P uptake also by other plants.

AMF have long been known to contribute to the P nutrition of plants (Smith and Read 2008). Less is known about the interaction between root exudates and AMF. Shibata and Yano (2003) have studied the P uptake by pigeon pea from aluminium phosphate and the role of AMF. In a compartment experiment which separates the P source from the roots it was tested with an air gap whether the root exudates are transported by the hyphae or along the hyphae. They could show that the P uptake was highest when root exudates were able to move through the soil without the gap and in presence of AMF inoculation.
These experiments inspired us to study the facilitation in pigeon pea - finger millet intercropping, which is a common intercrop system in southern India. Pigeon pea is generally a popular crop used for mixed cropping with 65 mixtures recorded in India (Ahlawat et al. 2005). Both crops have low requirements in nutrients and are cultivated on marginal lands. The combination of the two has been shown to be superior to monocropping (Mathimaran et al., in preparation). Following questions were addressed by our experiment:

I. Can pigeon pea take up P from insoluble forms such as $FePO_4$?
II. Can the root exudates of pigeon pea influence the uptake of P by finger millet?
III. Is finger millet able to take up P from $FePO_4$ on its own?
IV. What is the role of mycorrhiza for P uptake and competition between the two plants?
V. Does mycorrhization change the root length or ratio of thick to thin roots in the studied plants?

Methods

Experimental design

A two-compartment system was used with one compartment for pigeon pea and one for finger millet. Roots were separated from each other with a 32 µm nylon mesh (Fig 1). Containers were washed with detergent and rinsed with tap water, then they were sterilized by spraying with 70% ethanol and air drying. Pigeon pea was planted one week after germination and two one-week old finger millet seedlings were added after another month.

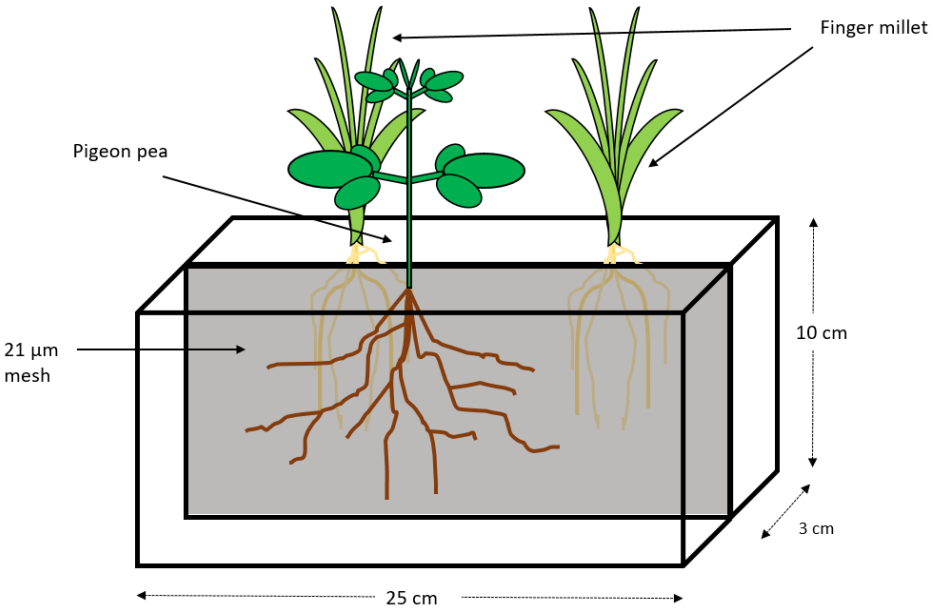

Figure 1: Design of the two-chambered microcosm. One pigeon pea plant was grown in one chamber and two finger millet plant in the second chamber. The two chambers are separated by a 21 µm nylon mesh which AMF but not the roots can pass.

The microcosms were systematically randomized in a weekly order. The main factors were P fertilization, AMF treatment and $FePO_4$ addition. $FePO_4$ is an insoluble form of P and cannot be taken up by most plants. Treatments with $FePO_4$ were conducted from March-May (n= 6) and treatments without $FePO_4$ were conducted from May-July (n=5). $FePO_4$ was applied at 100mg to the appropriate system, which was found to fertilize pigeon pea best (Ae et al., 1990). Three treatments with a monocrop of finger millet were undertaken with two finger millet seedlings planted in both compartments: $FePO_4$, $FePO_4$ + Myc and a control without any additions (n=4). Since the P solubilizing activity of finger millet is unknown we wanted to know whether finger millet alone can benefit from FePO4 addition.

After one month the systems received either full Hoagland solution (20ml with 2.72mg P) or Hoagland solution without P (Gamborg and Wetter 1975). The temperature in the greenhouse during the experiment ranged from 21-35°C.

Seeds and mycorrhizal inoculum

Finger millet (var. GPU28) seeds and pigeon pea (var. TTB-7) seeds were chosen for this study (Ankur Seeds Pvt. Ltd, Bangalore, India). The surface of the seeds were sterilized by 30 s in ethanol 96% and 2 min in NaClO 5% (commercial bleach), then washed by 0,01N HCl and washed 8 times with sterile water (Somasegaran and Hoben 1985). Seeds were pre-germinated in the substrate and covered with sand. Seedlings of same sizes were selected for transplanting them into the experimental pots according to the experimental design (see below). Before seeding, mycorrhizal inoculum containing *Rhizophagus fasciculatus* (53 spores/g) was applied to the planting hole of pigeon pea at 0.25 g. AMF inoculum was maintained in pot cultures with leek as a host plant and spore numbers were counted after they were isolated with a sugar gradient (Talukdar and Germida 1993). Controls received the same amount of substrate of the inoculum (9 parts Terragreen and 1 part Loess (from Biel-Benken, Switzerland)). All pigeon pea plants received, in addition, an inoculation with *Bradyrhizobium* sp. (DSMZ-5969, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany). Concentration of the inoculum was 0.3366 g/L cells and each seedling received 2 ml.

Control microcosms were supplied with microbial wash from the applied inoculum. 10 g Inoculum was filtered with 130 ml sterile water through a folded paper filter (Schleicher and Schuell, LS 14 ½) and 10 ml of the filtrate was applied to the soil. Aphids were present in the first weeks of growth and plants were sprayed with Zenar and Plenum (Syngenta Agro AG).

Substrate

A P deficient substrate was chosen to simulate P levels of tropical Ultisols of south India where the intercropping of finger millet and pigeon pea is practised. Expanded montmorillonite (Sorbix Premium oilbinder, Chem-Sorb, Maagtechnic AG, Switzerland) was washed with 0.1M HCl and kept for 16h in acid. Thereafter ChemSorb was washed with distilled water to remove acidity to a level of pH 5. Total P content was then reduced to 35.2 mg/kg (extraction with nitric acid and hydrochloric acid (1:3) and analysis via ICP-OES). Sterile acid washed ChemSorb was mixed with sand at 1:3. (<1 mg/kg P). Final water holding capacity was 37 %.

Harvest and data collection

Plants were harvested three months after planting (13 weeks pigeon pea, 9 weeks finger millet). Roots and shoots were separated and the fresh weight was recorded. Roots were thoroughly

washed, nodules were counted and samples taken to count root colonization. They were then stored in plastic bags at 4°C before they were scanned for root length analysis. Both roots and shoots were dried at 80°C for 24h and then weighed again. Dried shoots and roots were ground to a fine powder at 30Hz using a mixer mill (MM2224, Retsch, Haan, Germany).
P content of shoots and roots was measured using the molybdate blue method and transmission was measured at 827nm on a Shimadzu UV-160 spectrophotometer (Shimadzu Biotech, Duisburg, Germany) after acid digestion (Murphy & Riley, 1962).

AMF colonization
Root samples were taken at harvest. The roots were washed and cut into pieces of 1cm. They were then bleached and stored in 10% KOH at 4°C and in the case of pigeon pea afterwards cooked for 8 min at 90°C in a water bath. They were then stained with trypan blue (0.05% lactic acid, glycerol, water 1:1:1) for 15m at room temperature. After destaining in water they were examined for possible colonization of AMF (Phillips and Hayman 1970). Proportion of roots colonized by AMF hyphae, arbuscules and vesicles was calculated after Brundrett and McGonigle 1994, examining 100 intersections on 25 randomly chosen root pieces for each root sample.

Root length measurement
All roots were stored in plastic bags at 4°C after harvest to be scanned (Regent instruments LC4800P) for root length measurement with WinRHIZO 2015a (Regents Instruments, Canada). Roots of pigeon pea were separated in two equal portions and finger millet roots were scanned for each plant separately. The values of these two portions were then added for further analysis. Each scan was performed twice to account for possible error in the image analysis with WinRHIZO. Image analysis was then conducted with the tools provided by WinRHIZO.

Statistics
SPSS was used for statistical analysis. Three replicates of three different treatments were excluded because of experimental error during the fertilization and erroneous values. One-way ANOVA was used and Tukey test was used as a posthoc test to analyze for differences between subgroups.

Results
Pigeon pea biomass was significantly higher in the presence of $FePO_4$ (shoots: 2.92 g vs. 1.17

g, roots: 1.19 g vs. 0.75 g) and even higher in the presence of AMF symbiosis (shoots: 4.89 g vs. 1.92 g, roots: 1.69 g vs. 1.19 g) (Fig 2). The $FePO_4$ addition increased also the P uptake (shoot content: 3.31 mg vs. 0.58 mg, root content: 0.60 mg vs. 0.19 mg) and with AMF inoculation P uptake was more than doubled (shoot content: 7.60 mg vs. 1.47 mg, root content: 1.32 mg vs. 0.53 mg) (Fig 3). No significant increase by AMF inoculation was found when no $FePO_4$ was added. The P concentration in the shoots was not increased by any of the treatments (Fig S1) but in the roots similar results were found as for the P content with the significantly highest concentration with $FePO_4$ addition and AMF with and without P fertilization. Furthermore the treatment with $FePO_4$ addition and P fertilization had a significantly higher concentration than its counterpart without $FePO_4$ addition. In the treatments without $FePO_4$ addition the AMF treatment had significantly more P concentration than the one fertilized with P (Fig S2). Pigeon pea roots showed a colonization level between 10 and 15 % (Fig 6). P fertilization had no effect in either comparison.

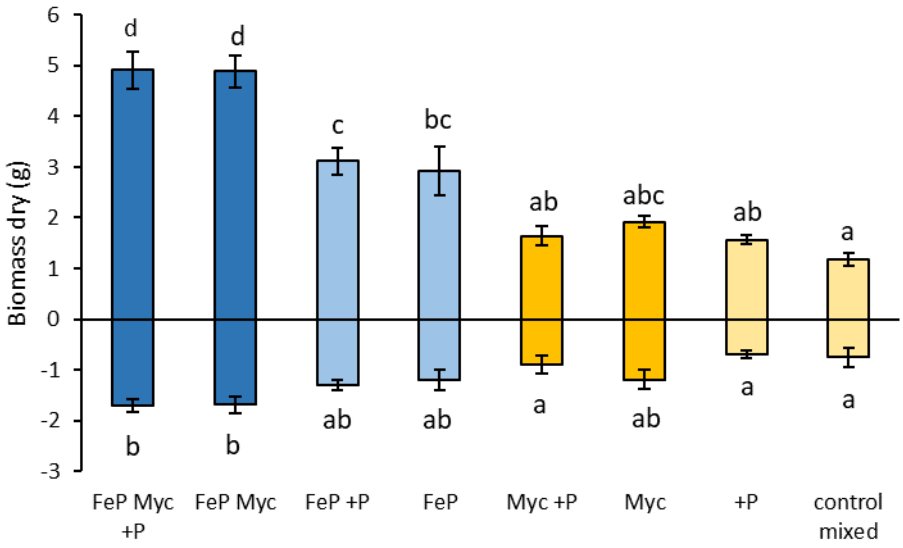

Figure 2: Biomass of pigeon pea, above and below ground. FeP stands for the addition of FePO4, Myc for mycorrhizal treatments, +P for the addition of phosphate fertilization and control mixed has received nothing. Tukey test was conducted within each shoot and root respectively. The values represent the mean ± SE of five replicates for the treatments with $FePO_4$ and four for the treatments without $FePO_4$. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

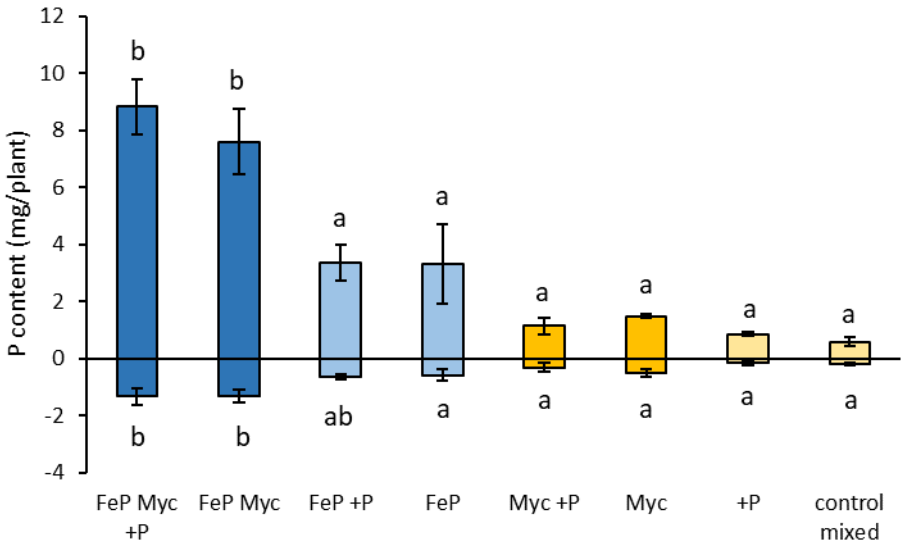

Figure 3: P content per plant of pigeon pea, above and below ground. FeP stands for the addition of FePO4, Myc for mycorrhizal treatments, +P for the addition of phosphate fertilization and control mixed has received nothing. Tukey test was conducted within each shoot and root respectively. The values represent the mean ± SE of five replicates for the treatments with $FePO_4$ and four for the treatments without $FePO_4$. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

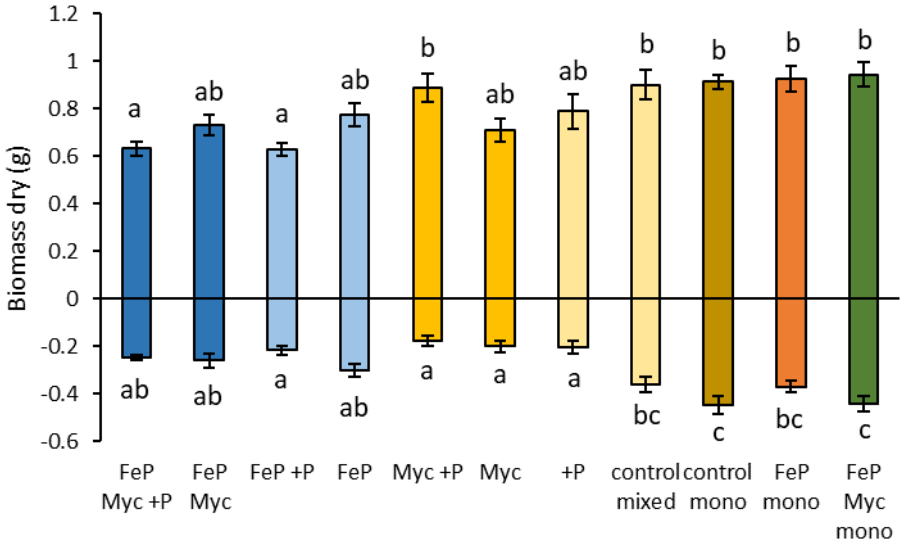

Figure 4: Biomass of finger millet, above and below ground. FeP stands for the addition of FePO4, Myc for mycorrhizal treatments, +P for the addition of phosphate fertilization, mono/mixed the planting of FM in both compartments or only one compartment with the other compartment planted with pigeon pea and controls have received nothing. Tukey test was conducted within each shoot and root respectively. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$, five for the treatments without $FePO_4$, and four for the treatments with monocropped finger millet. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

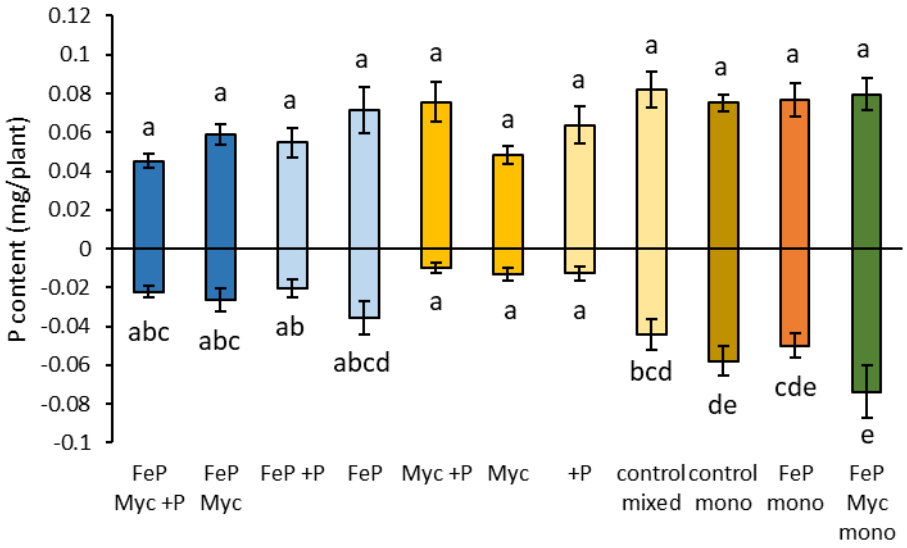

Figure 5: P content per plant of finger millet, above and below ground. FeP stands for the addition of FePO4, Myc for mycorrhizal treatments, +P for the addition of phosphate fertilization, mono/mixed the planting of FM in both compartments or only one compartment with the other compartment planted with pigeon pea and controls have received nothing. Tukey test was conducted within each shoot and root respectively. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$, five for the treatments without $FePO_4$ and four for the treatments with mono-cropped finger millet. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

When finger millet was grown with pigeon pea in the other compartment no major differences were found between P fertilization of AMF inoculation. Only when inoculated with AMF and with the addition of P and when nothing was added (control) could we find a significant difference in the shoot biomass to $FePO_4$ addition with P fertilization and with or without AMF inoculation. For the root biomass only the control without any additions was significantly larger than the other treatments without $FePO_4$ and the one with $FePO_4$ addition and P fertilization. When finger millets were grown in both compartments no significant differences between $FePO_4$ addition, mycorrhization or P fertilization were found in the root or the shoot weight of finger millet. However when comparing with the microcosms with pigeon pea in one compartment the same treatments as above ($FePO_4$ addition with P fertilization and with or without AMF) were significantly smaller for their shoot biomass. For the root biomass the treatments without any addition (control) and the one with AMF inoculation and $FePO_4$ addition were significantly larger than all roots when they shared the system with pigeon pea, except for the control which was on par. In the root biomass also the treatment with $FePO_4$ addition with two finger millet chambers was significantly larger than the treatments without $FePO_4$ (except the control) and the one with $FePO_4$ and P fertilization with pigeon pea in the other chamber (Fig 4). In the shoot of finger millet no significant differences could be found in the P uptake. In the roots, when pigeon pea was growing in the other chamber, only in the control without

any additions was there significantly more P taken up than in the other treatments without $FePO_4$ addition. When finger millet was grown in both chambers no significant difference were found within this group. But the same treatments when pigeon pea was grown in the other chamber were significantly smaller and also the one with $FePO_4$ addition and P fertilization. In the systems with only finger millet the treatments without any addition (control) and the one with AMF inoculation and $FePO_4$ addition had more P taken up in the roots than all systems shared systems with pigeon pea except for the one with $FePO_4$ addition (Fig 5). The roots in the systems with only finger millet showed no colonization (Fig 7). The difference was more pronounced in the roots than in the shoots and only significant in the roots for $FePO_4$ and AMF (Fig S3 and S4). Finger millets were only colonized in the presence of pigeon pea (between 5 and 10%).

The roots of the treatments with $FePO_4$ addition and the one with P fertilization without $FePO_4$ addition were assessed for both plants in the mixed microcosms. No effects of treatments were found in the root length (Fig S5 and S6), ratios of thinner and thicker roots or total diameter. Only pigeon pea and finger millet roots differed clearly in the diameter and root length.

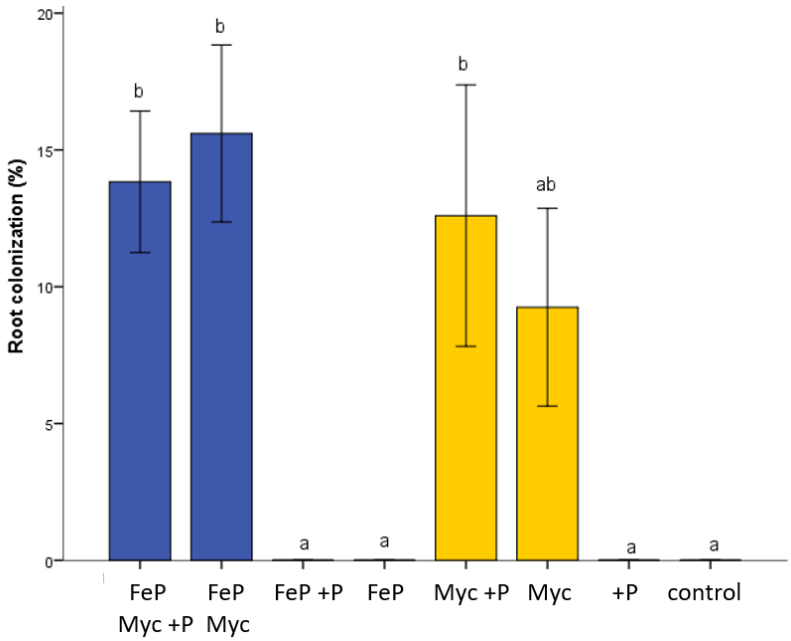

Figure 6: Root colonization of pigeon pea. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$ and five for the treatments without $FePO_4$. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

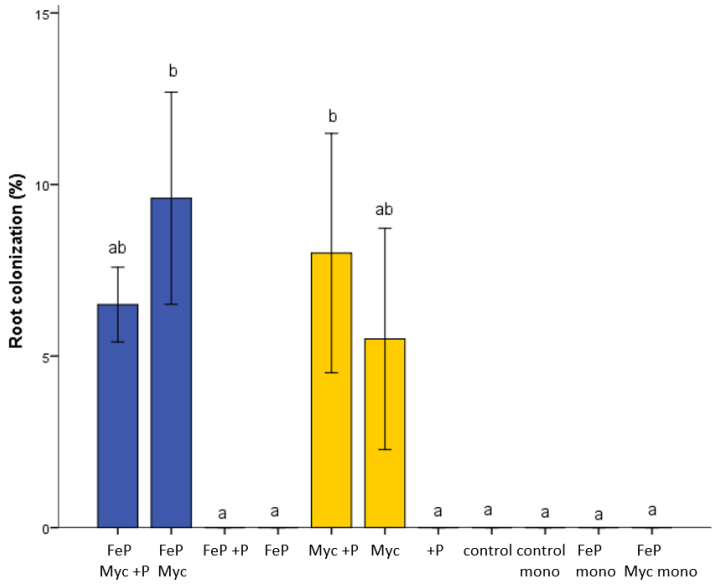

Figure 7: Root colonization of finger millet. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$, five for the treatments without $FePO_4$ and four for the treatments with mono-cropped finger millet. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

Discussion

The results are in line with the previous finding that pigeon pea is able to solubilize $FePO_4$ and respond to it with higher growth. Also confirmed was that AMF inoculation increased the effect greatly (Ae et al. 1990; Shibata and Yano 2003). Furthermore it was found that finger millet does not benefit from FePO4 addition and seems to be unable to solubilize $FePO_4$. However no facilitation of finger millet through the presence of pigeon pea and its P solubilizing ability was found. Instead finger millet had more biomass and higher P content in the roots when finger millets were grown in both chambers of the microcosm. Furthermore we could not confirm findings of other studies which found reduced length of tap roots and 1st and 2nd order lateral roots in kidney bean (Isobe et al. 2002) and a reduced total length in trifoliate orange by mycorrhization (Yao et al. 2009).

Interestingly finger millet was only colonized in the intercropped treatments. Mycorrhization is reduced below a certain level of P in the soil (Lekberg and Koide 2005). It may indicate that AMF were well supplied with P by the solubilizing activity of pigeon pea allowing them to establish symbiosis with finger millet. Yet this did not result in a growth increase. These results are in agreement with previous studies showing that AMF improved growth of legumes more when competing with grass species (Scheublin et al. 2007; Wagg et al. 2011). Other studies have also shown that competition for P favors the larger and older plants (*Cucumis sativus*) over smaller and younger seedlings (*Solanum lycopersicon*) (Merrild et al. 2013) which is also

the case here with pigeon pea being one month older than finger millet. Mechanisms underlying common mycorrhizal networks in plant competition are highly complex (Merrild et al. 2013). Facilitation and/or competition may depend on the identity and diversity of AM fungal species (van der Heijden and Horton 2009), the age and size of the plants (Walder et al. 2012), and the supply levels of important nutrients. However in our systems finger millets were increasingly shaded with the growth of pigeon pea and this may have had also an effect on these plants by reducing their photosynthesis. The results of this experiments are difficult to interpret further, because differences in treatments were small.

The fertilization was adjusted to the amount of P which would be given our field trials in Bangalore, India. Apparently this was too little to have an effect. We believe that unexpected processes of P absorption in our acid washed substrate are one reason for these unclear results. Probably the substrate immobilized the P fertilization by binding it to the clay particles in the expanded montmorillonite. It would have been easier to know of the fate of P by using radioactive labels with $FePO_4$ (^{32}P or ^{33}P) as they can be detected with higher accuracy and give a more detailed picture of these processes. Future studies may also analyze different fractions of P in the soil and how they change through intercropping and AMF inoculation.

Conclusions

We conclude that the capability of pigeon pea to solubilize immobile forms of P was of no benefit to finger millet. AMF did play a big role for an increased uptake of immobile P in pigeon pea but AMF seems to not have transported any P to finger millet. Another crop combination with pigeon pea may be able to benefit from this capability.

Supplement

Table S1: Details of root colonization with fungal organs. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$, five for the treatments without $FePO_4$ and four for the treatments with mono-cropped finger millet. PP= pigeon pea; FM= finger millet

	PP hyphal colonization	PP arbuscules	PP vesicles	FM hyphal colonization	FM arbuscules	FM vesicles
FeP Myc +P	15.7±3.9	12.3±2.7	1.5±1.0	5.8±1.5	3.8±1.2	1.0±0.6
FeP Myc	13.3±2.9	10.0±3.4	2.8±0.5	11.3±3.4	3.5±1.7	1.8±1.0
FeP +P	0	0	0	0	0	0
FeP	1.8±2.0	1.7±2.0	0	1.2±1.4	0	0
Myc +P	10.6±6.1	9.0±6.4	0	10.0±3.9	6.0±2.5	0
Myc	9.3±3.6	7.3±3.2	0.8±0.8	1.8±0.9	0	0
+P	0	0	0	0	0	0
Control mixed	0	0	0	0	0	0
Control mono				0	0	0
FeP mono				0	0	0
FeP Myc mono				0	0	0

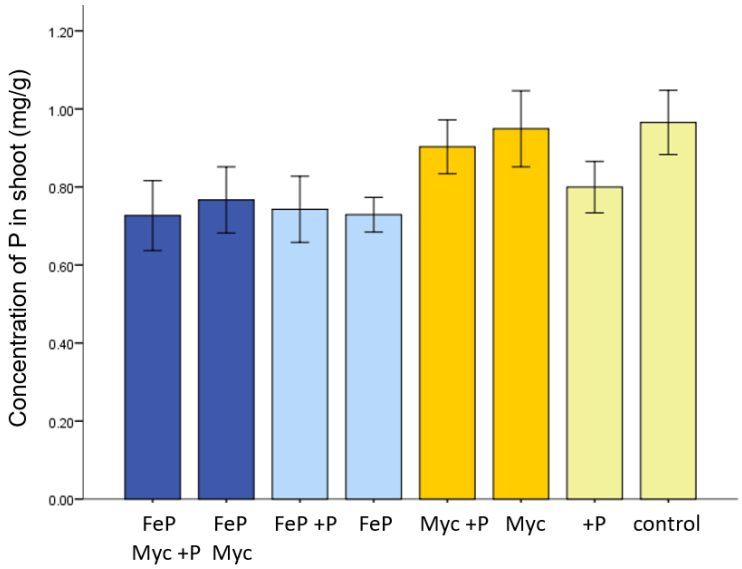

Figure S1: P concentration in dry shoot biomass of pigeon pea. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$ and five for the treatments without $FePO_4$. No significant differences were found.

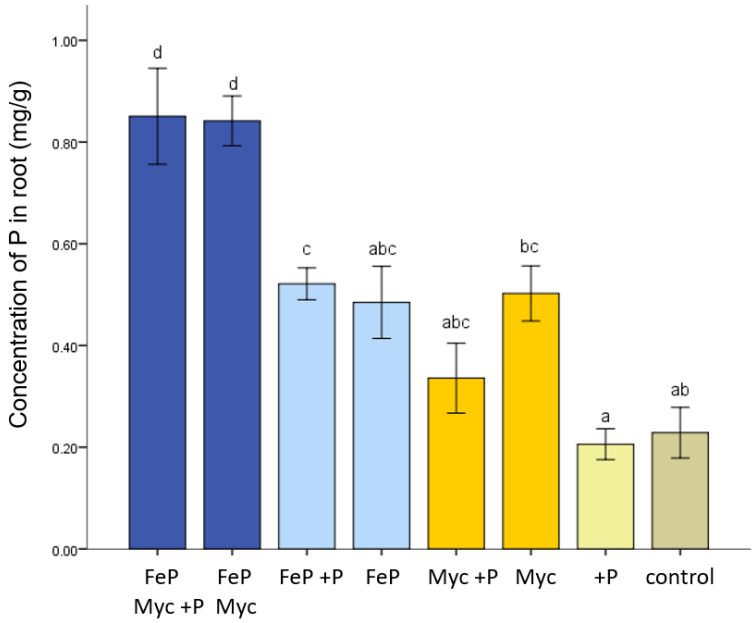

Figure S2: P concentration in dry root biomass of pigeon pea. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$ and five for the treatments without $FePO_4$. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

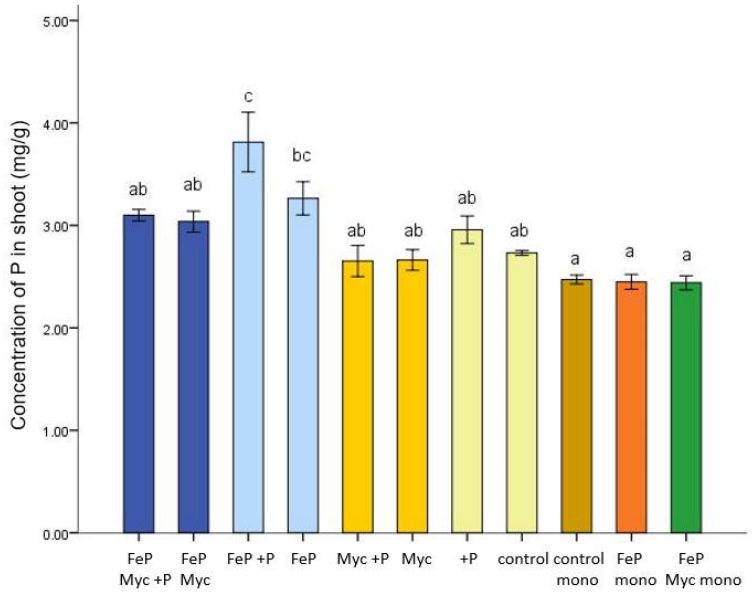

Figure S3: P concentration in dry shoot biomass of finger millet. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$, five for the treatments without $FePO_4$ and four for the treatments with mono-cropped finger millet. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

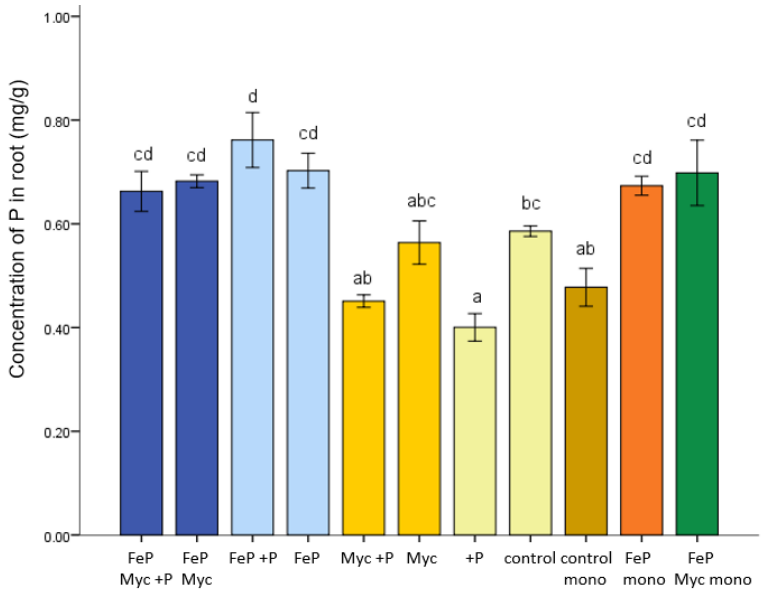

Figure S4: P concentration in dry root biomass of finger millet. The values represent the mean ± SE of six replicates for the treatments with $FePO_4$, five for the treatments without $FePO_4$ and four for the treatments with mono-cropped finger millet. Bars labeled with the same letter are not significantly different, according to Tukey-HSD ($p < 0.05$), after ANOVA.

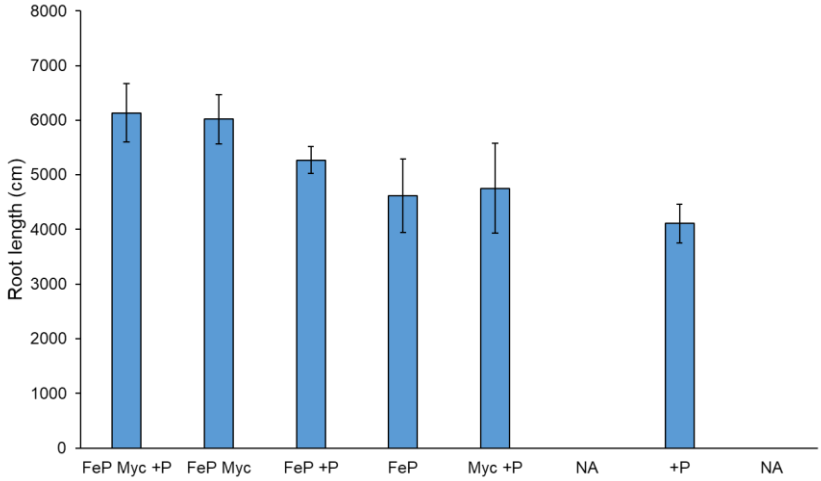

Figure S5: Root length of pigeon pea assessed with WinRhizo. The values represent the mean ± SE of six replicates for the treatments with FePO4, five for the treatments without FePO4. No significant differences were found after Tukey-HSD ($p < 0.05$) and ANOVA.

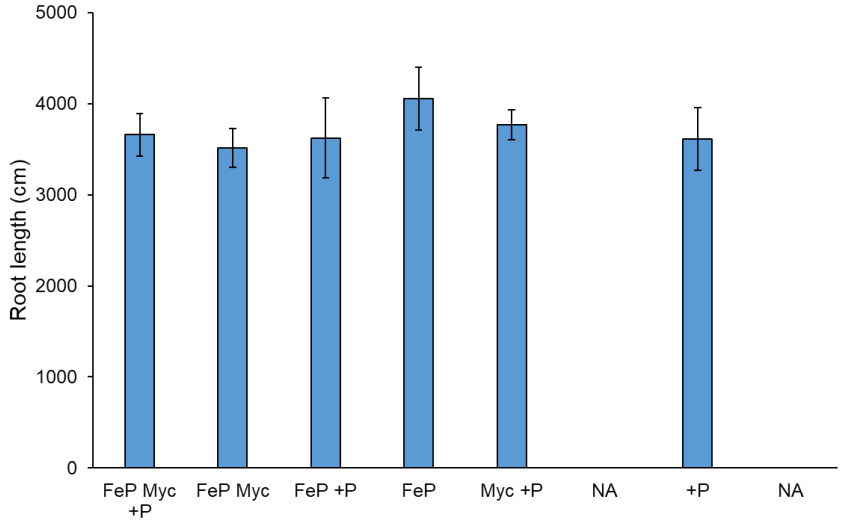

Figure S6: Root length of finger millet pea assessed with WinRhizo. The values represent the mean ± SE of six replicates for the treatments with FePO4, five for the treatments without FePO4. No significant differences were found after Tukey-HSD ($p < 0.05$) and ANOVA.

The following study has been conducted by me, Lukas Schütz, and I am the main author.

Tracing of an arbuscular mycorrhizal fungus applied to a mixed cropping system of pigeon pea (*Cajanus cajan*) and finger millet (*Eleusine coracana*)

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Abstract

Arbuscular mycorrhizal fungi (AMF) have a great potential to make agriculture more sustainable. Industrial agriculture depends highly on external inputs like fertilizers and pesticides, which may disturb global nutrient cycles and harm the environment. AMF may be used as biofertilizers, improving nutrient use efficiency and crop yields without unwanted side effects. To trace the spread and survival of an AMF strain applied as biofertilizer, and to study its potential effects on the indigenous AMF community, it is important to develop and apply DNA markers with a high power of discrimination. A recently published method, based on single molecule real-time (SMRT) sequencing, allows highly resolving and specific profiling of AMF communities, based on AMF-specific PCR primers that amplify a ca. 1.5-kb long fragment of ribosomal DNA covering parts of SSU, ITS and parts of LSU (Schlaeppi et al., New Phytol. 212, 780-791, 2016). We applied this new technique to DNA extracted from rhizosphere soil from a field trial in South India, in which two AMF species (*Rhizophagus fasciculatus* and *Glomus leptotichum*) and one PGPR (*Pseudomonas fluorescens*) had been used to inoculate finger millet and pigeon pea, monocropped and in mixed cropping. Although the amplification of ribosomal DNA and the processing of the barcoded samples on the PacBio platform was successful, it became evident, after processing the reads that the amount of quality reads in our samples was low. However some sequences belonging to Glomeromycota from the inoculum of *Rhizophagus fasciculatus* could be found also in DNA sampled from the field sites and provide at least a proof of concept. Most field samples contained amplicons which belonged to Glomeromycota and belonged to different species of AMF of the Glomeromycota, but the

success of sequencing differed over years and also within the treatments, thus no conclusions about changes in the AMF community could be drawn.

Introduction

The highly mechanized, industrial agriculture has reached its limits in many aspects. Monocultures, managed by herbicides and pesticides, harbor little space for other organisms, above and belowground (Wezel et al. 2014). Excessive application of mineral fertilizers lead to low nutrient use efficiency, cause pollution of the nearby environment and may even change global nutrient cycles (Kahiluoto et al. 2014).

Microbial inoculants, so-called biofertilizers, are a promising technology to reduce the use of conventional inorganic fertilizers. Rhizosphere microorganisms are either growing in the rhizosphere or as endophytes inside the roots and can serve as biofertilizers as they are able to fix nitrogen (N), help to access nutrients such as phosphorus (P) and N from organic fertilizers and soil stocks, improve drought tolerance, improve plant health or increase salt tolerance (Vessey 2003; Lekberg and Koide 2005; Arora 2013). Arbuscular mycorrhizal fungi (AMF) are one of these microorganisms, which are obligate biotrophs. AMF support plant growth directly by enhancing nutrient (P, Zn and Cu) and water uptake, thereby receiving carbohydrates from photosynthesis (Smith and Read 2008). With their extra-radical hyphal network they are able to explore more soil volume than plant roots alone. AMF also support plant growth indirectly by improving soil structure and resistance to certain root pathogens (Treseder and Turner 2007; Smith and Read 2008).

Mixed cropping increases the diversity in agriculture of the crop itself and also the biodiversity of animals, arthropods and microbes (Brooker et al. 2015). At the same time it increases yields in the sum of all crops included on a given piece of land. Such yield increase is called overyielding. One reason are interspecific plant interactions which change the use of resources (Vandermeer 1989; Zhang and Li 2003). Mixed or intercropping was the general practice in traditional agriculture and vanished only in the wake of the highly mechanized, industrial agriculture (Altieri 1999; Lithourgidis et al. 2011). Pigeon pea-finger millet intercropping is still common practice in southern India. Pigeon pea is generally a popular crop used for mixed cropping with 65 mixtures recorded in India (Ahlawat et al. 2005).

Determination of success of field inoculation with AMF always lacked one important parameter: It was not possible to trace back the inoculum which was added to the soil. With the single molecule real-time (SMRT) by Pacific Biosciences sequencing it is possible to sequence long amplicons with low error rates because the amplicon is circularized allowing the

polymerase to pass multiple times thereby reducing the relatively high error rate of single-pass reads (Schlaeppi et al. 2016). The ability to sequence long amplicons is necessary to have enough resolution to distinguish strains of AMF and thereby identifying the inoculated strain from the indigenous AMF community. Although successfully used for other fungal communities, the amplification of the ITS region in the ribosome alone has a low recovery of Glomeromycota sequences. But with the ability to sequence longer amplicons, the amplification of a ribosomal fragment spanning partial SSU, the ITS and partial LSU has a higher resolution of the Glomeromycota (Schlaeppi et al. 2016). The inoculation with AMF may influence the diversity of the indigenous AMF community and other microbial life. Microbial communities may change for less diversity and less functionality but also win-win combinations for plant growth of AMF and "helper" bacteria are well-known (Vessey 2003). Furthermore AMF can influence the chemical composition of root exudates, which in turn are a major nutrient source for the bacteria in the rhizosphere (Harris 2009). Understanding more about the influence of AMF inoculations on microbial communities and AMF communities alone is now with the new sequencing methods possible.

Here, we present an analysis of AMF communities in rhizosphere samples from a large field experiment designed to tested the effect of three different inocula (AMF alone, PGPR and in combination), the effect of intercropping pigeon pea and finger millet and the variation of two locations in India with different agro-climatic zones in two subsequent years. Our hypothesis was that each of these factors will also have an influence on the AMF community composition. The technical goal of this investigation was to trace back the inoculum at harvest time.

Methods

Preparation of DNA from selected soil and inoculum samples

The soil that was analyzed in this study has been sampled from a bigger study on the application of PGPR and AMF in pigeon pea- finger millet intercropping in two field sites. A more detailed descriptions can be found in the supplement. To test for survival of the AMF inoculum at harvest time, and to analyze the indigenous AMF community we planned to compare each inoculation (no inoculum, PGPR, AMF and the combination of AMF and PGPR) at 50% of the recommended dose of mineral fertilization (25:50:25 NPK (elemental form) kg ha^{-1}). These treatments had been replicated from each of the two years field trial and the two sites Kolli Hills and Bangalore (details of the samples can be found in the electronic supplement S3). Five randomly selected plants of each species in a plot were uprooted and the soil adhering to the roots was collected, thereafter referred to as rhizosphere soil. The soil was then pooled with the

other three replicate plots. Thus each sample represents 4 pooled field replicates. We first tested DNA extracted from the roots contained in the samples, but for unknown reasons, mostly there was no amplification with the primers used. Any changes to the annealing temperature of the primer, DNA or primer concentrations were unsuccessful. We therefore switched to DNA extracted from the rhizosphere and got bands from most samples. Rhizosphere samples had been shown previously to contain more DNA from AMF species and thus yielding a better amplification (Schlaeppi et al. 2016). In a few samples, though, DNA was amplified again because yields were too low, or 1µL original DNA was preamplified with the unspecific Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, PA, USA)(details in the electronic supplement S3).

DNA was extracted from 250 mg of rhizosphere soil samples using power soil DNA kit (MOBIO laboratories Inc., USA) following the manufacturers protocol (bead beating and spin column filter extraction). The soil sample was added to the power bead tubes containing the suspension buffer (C1) and lysis buffer (C2) were added before vortexing at maximum speed, where lysis occurs by mechanical and chemical methods. Then the samples were treated with PCR inhibitor removing solution (C3) provided by the manufacturer to remove contaminants. The total DNA bound using binding solution (C4) to the silica membrane provided in the spin column is then washed (C5) and eluted with 50 µl of solution C6 from the membrane.

Both AMF species (*Rhizophagus fasciculatus* and *Glomus leptotichum*) were maintained in pot cultures with leek as a host plant at the Centre for Natural and Biological Resources and Community Development (CNBRCD), Bangalore, India, and spores from the soil were isolated with a sugar gradient (Talukdar and Germida 1993).The DNA of inoculum itself was extracted from the spores (Fig S2) by crushing 20 spores, addition of 5 mM Tris-HCl, pH 8.5 and centrifugation to pellet solid pieces. DNA was also extracted from the roots (60mg) included in the inoculum mix with NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany). All DNA quantification was done with Qubit 2.0 fluorometer with the dsDNA HS assay kit (Thermo Fisher Scientific, MA, USA).

Amplification and sequencing

For the amplification of the AMF specific sequences we largely followed the paper by (Schlaeppi et al. 2016). The same fused Krüger primers ‘SSUmCf’ and ‘LSUmBr’ (Krüger et al. 2009) containing wobble bases were used. However we used different shorter 8-digit barcodes to identify the samples in the libraries (complete primers in Table S2). They were created with the suggestions from Faircloth and Glenn (2012) and taken from their

supplementary File S7 from the groups ed4 and ed5, securing from 4 or 5 base mutations during amplification. Barcoded PCR primers were synthesized (HPLC purified grade) at Microsynth (Balgach, Switzerland). For amplification we used the same Phusion High-Fidelity DNA polymerase system however from a different producer (NEB, Ipswich, MA, USA) and the same composition of the PCR reaction mix. Because of the sometimes low quantity of the amplicons, samples were amplified in 4 separate 50µL PCR reactions with each 5 µL of original DNA. By having technical replicates this also reduces the random increase of some sequences picked up at the beginning of the amplification process which would give an unrealistic picture of the AMF community. Thermal cycling was performed on an Sensquest lab cycler (Sensquest GmbH, Germany) with the following conditions: 2 min initial denaturation at 98°C, 41 cycles of 10 s denaturation at 98°C, 30 s annealing at 60°C and 1 min elongation at 72°C and a final elongation of 10 min (settings: no hot start and temperature control by algorithmic method). Two lanes of a 2% Agarose gel were then loaded with each 100 µL (two pooled PCR reactions) and run for 80min at 70V. The band at about 1500bp was then visualized under blue light (not DNA damaging) and cut with a scalpel which was sprayed with ethanol and flamed with a lighter before every new band was excised to prevent cross contamination. The Gel and PCR reaction clean-up' kit (Macherey-Nagel, Düren, Germany) was then used to extract the DNA from the gel cuts. The two cuts were dissolved and loaded onto one column. DNA was eluted in 5 mM Tris-HCl, pH 8.5. Finally, we built an amplicon library by equimolar pooling of the barcoded DNA samples with each samples represented by 100ng.

The ligation of the amplicons with the hairpin sequencing adapters and subsequent clean-up with AMPure (Beckman Coulter Life Sciences, USA) and quality analysis (Bioanalyzer; Agilent, Santa Clara, CA, USA) were done at the Functional Genomics Center Zürich (Zurich, Switzerland; http:// www.fgcz.ch). There the libraries were sequenced in two cells on PacBio® RS II Instrument (p/n 100-210-100).

Sequence data processing

To process the sequences obtained we oriented ourselves to the scripts published in Schlaeppi (2016) and Schloss (2016). The 'reads of insert' (ROI) with ≥ 5 passes and ≥ 97% accuracy were extracted from the raw data with SMRT Analysis Software (PacBio) with the CCS protocol and processed to obtain quality sequences using MOTHUR (v.1.34.4; Schloss et al., 2009) and flexbar (Dodt et al., 2012). Quality sequences were clustered into operational taxonomic units (OTUs) at ≥ 97% sequence similarity with the UPARSE series of scripts (Edgar

2013). Reads were sorted by abundance, de-replicated and single-count and chimeric sequences were excluded for OTU delineation.

The OTU table and OTU-representative sequences are provided in the electronic supplement (S4 and S5). Taxonomic identities were assigned to the OTU representative sequences utilizing the UNITE database (dynamic clustering thresholds, release 02/09/2017; Koljalg et al. 2014) with BLAST in the QIIME environment (Caporaso et al. 2010). The UNITE taxonomy table is provided in the electronic supplement (S6). OTUs assigned to the phylum 'Glomeromycota' were subsequently queried against the AMF reference data set (Krüger et al. 2012) to obtain high-resolution and AMF-specific taxonomy (provided the electronic supplement S7). Importantly, the AMF reference set is based on the same rRNA operon fragment (pSSU-ITS-pLSU) that we amplified for community sequencing. For this task, we utilized uclust- based consensus taxonomy in the QIIME environment and a QIIME formatted version of the reference data set (electronic supplement S8a,b,c). The entire dataset was analyzed in R Studio version 0.99.491. The analysis comprised a first step (examination of all OTUs and UNITE taxonomy) followed by subsetting the investigation to Glomeromycota OTUs (with AMF-specific taxonomy). The OTU tables were normalized by the sampling depth of each sample and expressed the abundance of individual taxa as percentage relative abundance.

Due to the low amount of quality reads, we attempted to extract the 'reads of insert' (ROI) again. Although accompanied with a higher error rate of 11% (Korlach 2013) we filtered the ROI again with only 1 pass and 99% accuracy from the raw data with SMRT Analysis Software (PacBio) with the CCS2 protocol. Afterwards the same process as above was applied.

Results

Most rhizosphere DNA samples yielded amplicons of the expected size successfully, although for some samples the large reaction mix of 200 µL was really necessary to yield enough DNA for sequencing. Five samples showed no amplification (4 from first year, 1 from second year). Amplification from the root extracts of *R. fasciculatus* was successful but for the spore extract DNA had to be reamplified with GenomiPHI V2 DNA Amplification kit. For *G. leptotichum* only a smaller fragment of about 700 bp was amplified and no amplification was possible from the roots (Fig S1). Still we sequenced this smaller fragment again after reamplification with GenomiPHI V2 DNA Amplification kit (gelband 66G in fig S1). Interestingly the band disappeared after reamplification with the same settings as before (gelband 66A in fig S1). Details of how each treatment was the amplified can be found in the electronic supplement (S3) and gel electrophoresis pictures in Fig. S1.

Of the 63 samples, 52 samples were successfully amplified. Some minor difference between the two pooled replicates could be seen which shows the need to have technical replicates to counterbalance random amplification at the start of the PCR. Products of the PCR where then sequenced and separated by their barcode. The sequencing resulted, after the first 5-pass filtration, in 1430 reads in the first cell and 3470 in the second cell. After OTUs were assigned with UPARSE, results from the cells were combined, and 2850 sequences were left belonging to 348 OTUs (10.1% Chimeras). Most sequences were between 1300 and 1650 bp long (Fig 1). According to the UNITE database, 153 belong to Glomeromycota represented by 1820 sequences. The results of this dataset are presented in figure 2 and 3 and table 1.

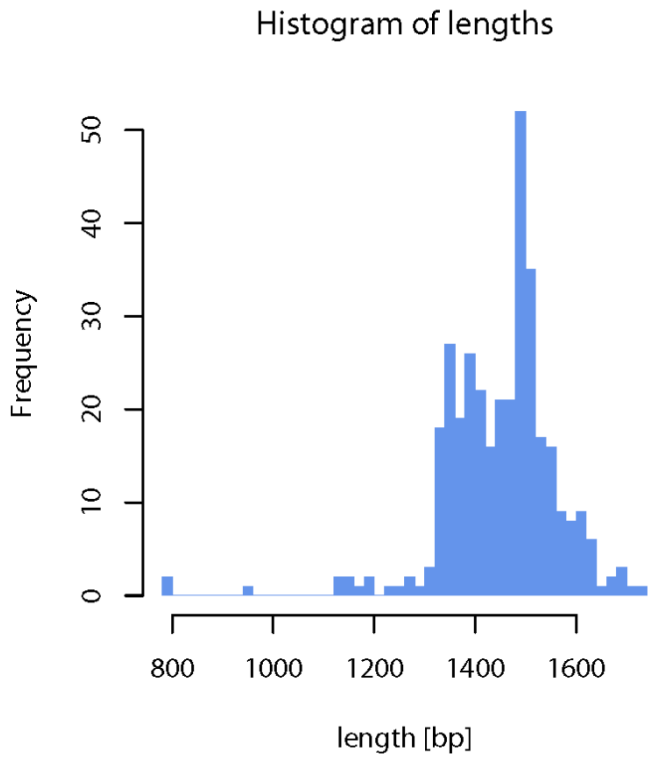

Figure 1: Lengths distribution of quality sequences. The frequency of the sequences lengths (in base pairs, bp) is plotted for the 3192 quality sequences. All these sequences were used for clustering the OTUs with UPARSE.

In an attempt to increase the amount of quality reads we also processed the raw sequence without the PacBio inherent proof reading (CCS). The sequencing resulted after the 1-pass filtration in 1305 reads in the first cell and 3535 in the second cell. After OTUs were assigned with UPARSE, results from the cells were combined, and 3192 quality sequences were left belonging to 302 OTUs (8.2% Chimeras). According to the UNITE database 121 belong to Glomeromycota, represented by 1862 sequences. It is unexpected to have less OTUs from 1-pass reads, but this may be due to the higher error rate here. The results of this attempt were then not further analyzed.

We were able to sequence and identify some OTUs from the inoculum in the high quality sequences of the 5CCS. Only one unique OTU was obtained for the spore extract of *Rhizophagus fasciculatus* which according to the UNITE database is identified as Glomus sp.. We were more successful with DNA extracted from the roots of the inoculum, and found 7 OTUs of which both OTU140 and OTU58 belong to Glomeromycota according to the UNITE database and represent strains of *Rhizophagus irregularis*. The other OTUs belonged to an unidentified *Sebacinales* species, *Cladosporium dominicanum*, *Cladosporium halotolerans* and two could not be assigned in the UNITE database. We could also find the glomeromycotan OTUs again each in two separate treatments of the first year field trial, OTU58 in intercropped pigeon pea inoculated by AMF+PGPR and OTU140 intercropped pigeon pea inoculated by only AMF, however in each sample they were only represented by one sequence. Both identifications were confirmed by the more specific Krüger reference datset and they were identified as *Rhizoglomus irregulare*, the newest taxonomic classification but synonymous with *Rhizophagus irregularis*. Uniquely with this database also OTU106 was identified as an AMF and assigned to *Claroideoglomus* sp., which could be found again in three samples of the second year field trial, namely monocropped pigeon pea inoculated by PGPR, monocropped pigeon pea inoculated by PGPR and uninoculated intercropped pigeon pea. From the spore extracted DNA of *Glomus leptotichum* we identified OTU16, according to UNITE database the Ascomycete *Cladophialophora modesta* and unassigned in the Krüger database, which was found in 7 treatments represented there by up to 6 sequences.

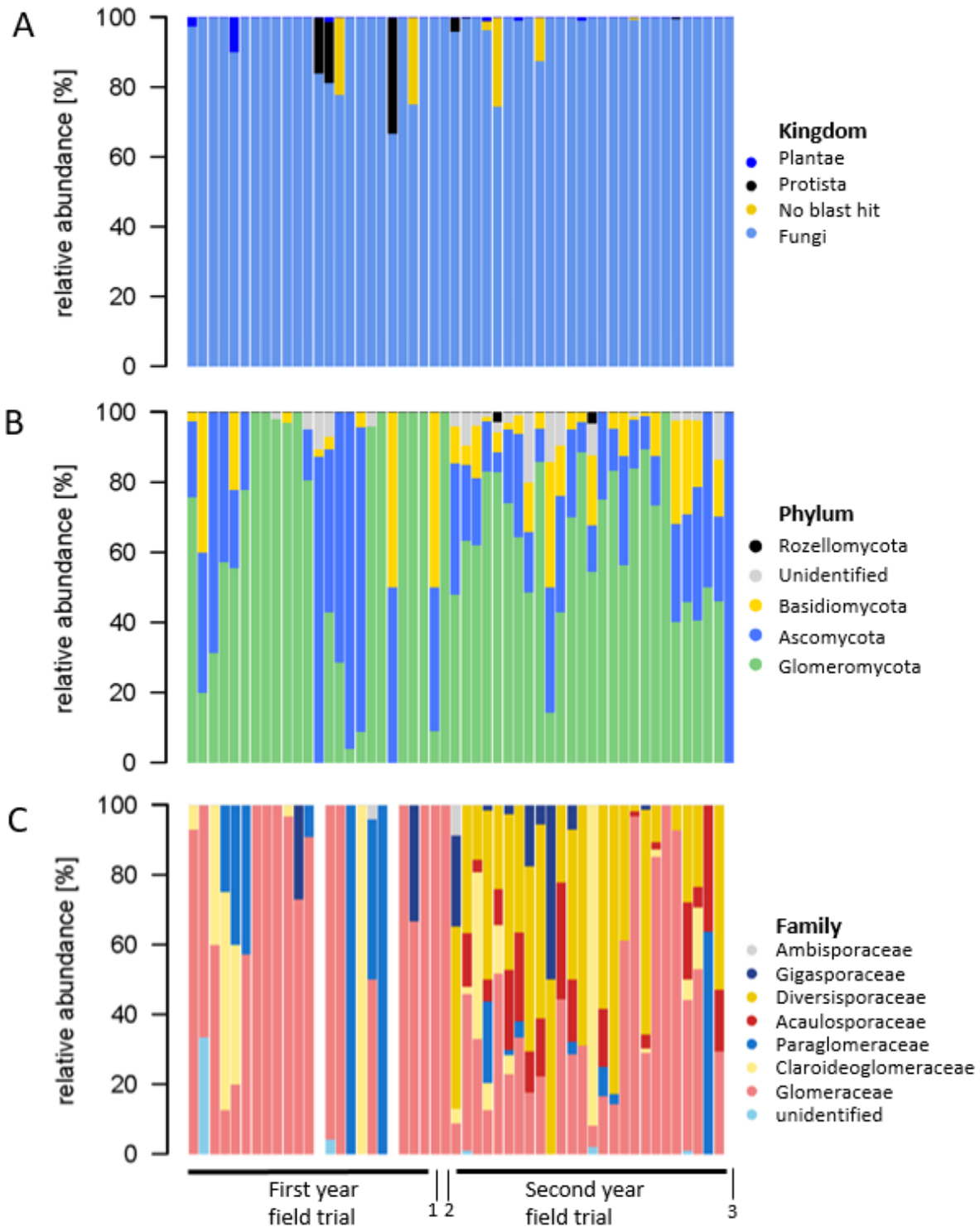

Figure 2: Taxonomic coverage with sequences that passed 5 CCS at 97% accuracy. OTU representative sequences were taxonomically assigned using BLAST against the UNITE database for broad taxonomic classification. The taxonomic composition in each sample is reported as relative abundance (RA) at (a) kingdom, (b) phylum and (c) family level. AMF community profiles (c) consist of Glomeromycota OTUs only. Sometimes percentage values are made up of very few sequences only, for absolute numbers of Glomeromycota see figure 3. Not all samples were sequenced, further details are given in table S1. Inoculum is displayed in 1 *Rhizophagus fasciculatus* extracted from spore, 2 *Rhizophagus fasciculatus* extracted from root and 3 *Glomus leptotichum* extracted from spore. Samples identity can be found in the last column in the electronic supplement S3.

The classification of amplicons is displayed in Fig. 2 according to the UNITE database. Most sequences belong to Fungi, but also some belonging to Protista and Plantae were amplified (Fig 2A). Of the fungal amplicons a large proportion was identified as Glomeromycota but also many Ascomycota and less Basidiomycota and Rozellomycota (Fig 2B).

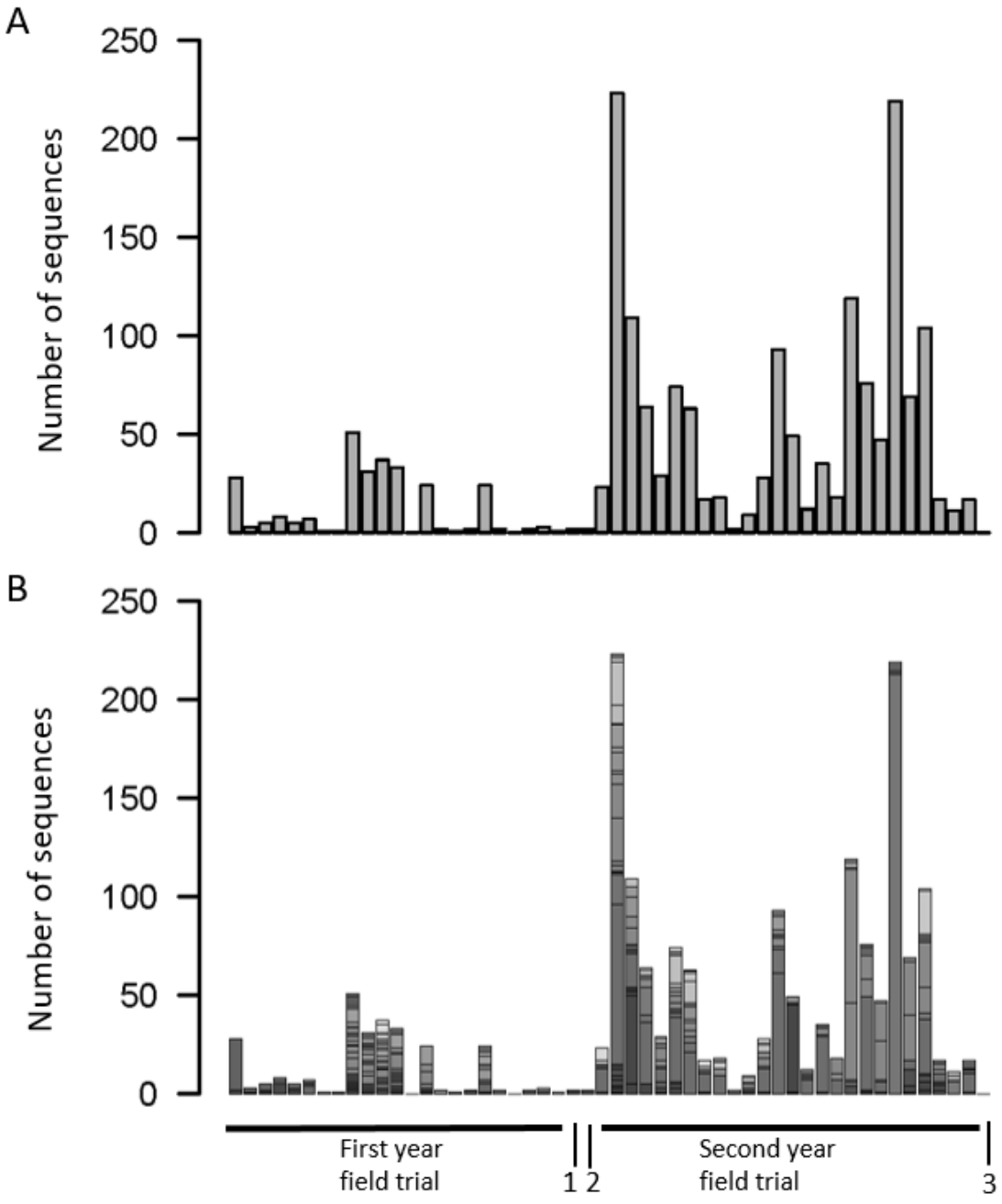

Figure 3: Nr of sequences belonging to Glomeromycota by treatments. (a) total numbers and (b) additionally split by nr sequences belonging to each OTU. Not all samples were sequenced, further details are given in table S1. Inoculum is displayed in 1 *Rhizophagus fasciculatus* extracted from spore, 2 *Rhizophagus fasciculatus* extracted from root and 3 *Glomus leptotichum* extracted from spore. Samples identity can be found in the last column in the electronic supplement S3.

The combination of the different primers by Krüger et al. (2009) into one with wobble bases comes with a cost of amplification of other sequences of fungi, protista or plants. In a few treatments the proportion of Glomeromycota was less than half, raising the question whether this cost reduces overall success of sequencing AMFs. Also the Glomeromycota sequences at the family level (Fig 2C) are depicted as proportions, however sometimes are made up of only few sequences each sample and treatment. The big majority of the first year samples belong to Glomeraceae, in the second year Diversiporaceae and Glomeraceae have almost equal proportions. Also for the total number of sequences belonging to Glomeromycota a big difference between first and second year samples was found (Fig 3A) with many more sequences in the second year. The diversity of OTUs was quite different in the treatments. Also in treatments with many sequences large proportions were only assigned to few dominant OTUs e.g. in AMF inoculated intercropped finger millet and monocropped pigeon pea at Kolli Hill site in second year field trials.

We have used two databases for taxonomic assignment. UNITE comprises well annotated fungal ITS sequences from identified herbarium specimens that include full herbarium reference identification data, collector/source and ecological data whereas the Krüger reference set is more specific for the length of the amplicon that was amplified. Since we did not trim our sequences some are also shorter than this fragment. A comparison of the different assignments to the family level of Glomeromycota is provided in Table 1. The accuracy is higher when the OTUs were assigned with the Krüger reference set and in this way more OTUs could be assigned to Glomeromycota.

Table 1: Comparison of the two databases UNITE and Krüger used for taxonomic assignment for the example at the family level of Glomeromycota.

Family	Nr of OTUs according to UNITE taxonomy	Nr of OTUs according to Krüger taxonomy
Acaulosporaceae	11	11
Ambisporaceae	NA	2
Archaeosporaceae	1	1
Claroideoglomeraceae	1	22
Diversisporaceae	11	11
Gigasporaceae	8	9
Glomeraceae	90	100
Paraglomeraceae	1	17

Discussion

The number of reads in our samples was low. Also Schläppi et al. (2016) reported problems with a polymerase, a Phusion Hot Start II DNA polymerase system, which was likely the cause for obtaining bad results. We used the Phusion polymerase by a different producer (NEB) than Schläppi et al. (2016) did. This is a possible reason for our sequencing result. Another possibility is that contrary to Schläppi et al. (2016) we did not clean up the gel-extracted DNA with the AMpure kit. This may have caused many small strands of DNA to be sequenced (Fig S3). But most reads were in the range of the target amplicon thus an incompatibility of the Phusion polymerase by NEB is more likely, but the exact reason cannot be identified.

Yet the successful tracing of at least the inoculum of *Rhizophagus fasciculatus* shows a proof of concept of PacBio sequencing for the study of AMF inoculation experiments. The "wrong" name of the OTU which was assigned to *Rhizophagus irregularis* may in fact not be wrong because the inoculum of *Rhizophagus fasciculatus* (formerly *Glomus fasciculatum*) was isolated more than 30 years ago and since then the taxonomy has changed many times in the Glomeromycota. The genera *Glomus* and *Rhizophagus* used to belong to the same genus (Redecker et al. 2013). The number of traced sequences was very low and thus the results not very reliable. OTU106, assigned to *Claroideoglomus* sp., was found in treatments where no AMF were inoculated. This could be an erroneous sequence, but since the inoculated strains are local strains from South India, they could also be present in the soil. We refrain from interpretations of the AMF community since the reads of the Glomeromycota were distributed so unevenly across the treatments and years.

Conclusions

The knowledge of the survival and tracing of AMF inoculants is still very important to understand AMF biofertilization better. As well how they influence the community of native AMF is still of high importance. In this case the sequencing would have to be repeated and another polymerase to be used for the amplification, also the clean-up of the gel-extracted DNA seems to be recommended.

Supplement

Origin of the field samples studied

The field samples analyzed in the current study came from a large field experiment done in the context of the ISCB BIOFI in the years 2015/2016 (Mathimaran et al., in preparation). The study sites were located at the University of Agricultural Sciences, GKVK campus, Bangalore and Kolli hills, Tamil Nadu state, India. The experiments were performed in a plot with four randomized field replicates per treatment in each location. Pigeon pea seedlings were grown in plastic bags in a nursery at the study site, under well-watered conditions, one month before the planned sowing of finger millet in the field to have ca. 28d-old seedlings ready for planting into the experimental plots at the beginning of the rainy season for crop growth. The inoculants were in case of AMF *Rhizophagus fasciculatus*, selected for finger millet, and *Glomus leptotichum*, selected for pigeon pea. They were amplified in pot culture with Rhodes grass *(Chloris gayana)* and the substrate (vermiculite based) with cut roots was applied to pigeon pea at the rate of 1g in the plastic bag at 24 spores g^{-1} substrate (*Glomus leptoticum*) and at the rate of 323 kg per ha to the furrow when sowing finger millet at a concentration of 15 spores g^{-1} substrate (*Rhizophagus fasciculatus*). The AMFs were obtained from Centre for Natural and Biological Resources and Community Development (CNBRCD), Bangalore. The PGPR strain was multiplied in King's B medium and the liquid culture consisting 1 x 109 CFU per ml of Pseudomonas sp. MSSRFD41, described in detail in (Sekar and Prabavathy 2014), was applied as seed coating at the rate of 5 ml per kg seed. Additionally, a band application (along the planting rows) was applied at the rate of 49.5 liters (consisting 1 x 109 CFU per ml) per 7.5 t FYM per ha). Rhizobium was applied as seed coating at the rate of 10ml per kg of pigeon pea seeds. They were tested at two fertilizer levels on the base application of 7.5 t FYM per ha in pigeon pea and finger millet mono cropping and pigeon pea-finger millet inter cropping system. The plot size was 25 m^2 with total layout area of 2059.2 m^2. Low bands of soil were constructed between the plots to inhibit water flow and to minimize cross-contamination of the inoculants.

Table S1: List of the adopted Krüger primers joined with their padding sequence, barcode and the fusion of all parts.

BarcodeID	PaddingBarcode_sequence	Sequence: 5´> 3´	Fusionprimer
forward Primers			
8F1	GGTAGTTGTTCCG	TATYGYTCTTNAACGAGGAATC	GGTAGTTGTTCCGTATYGYTCTTNAACGAGGAATC
8F2	GGTAGAACAACCG	TATYGYTCTTNAACGAGGAATC	GGTAGAACAACCGTATYGYTCTTNAACGAGGAATC
8F3	GGTAGAACACGAC	TATYGYTCTTNAACGAGGAATC	GGTAGAACACGACTATYGYTCTTNAACGAGGAATC
8F4	GGTAGACTGCCAA	TATYGYTCTTNAACGAGGAATC	GGTAGACTGCCAATATYGYTCTTNAACGAGGAATC
8F5	GGTAGAACAACCG	TATYGYTCTTNAACGAGGAATC	GGTAGAACAACCGTATYGYTCTTNAACGAGGAATC
8F6	GGTAGAACCGAGA	TATYGYTCTTNAACGAGGAATC	GGTAGAACCGAGATATYGYTCTTNAACGAGGAATC
8F7	GGTAGAGTCTGTG	TATYGYTCTTNAACGAGGAATC	GGTAGAGTCTGTGTATYGYTCTTNAACGAGGAATC
8F8	GGTAGAAGGTGGT	TATYGYTCTTNAACGAGGAATC	GGTAGAAGGTGGTTATYGYTCTTNAACGAGGAATC
Reverse Primers			
8R1	CCATCAACCAGGT	AACACTCGCAYAYATGYTAGA	CCATCAACCAGGTAACACTCGCAYAYATGYTAGA
8R2	CCATCAACCTCTC	AACACTCGCAYAYATGYTAGA	CCATCAACCTCTCAACACTCGCAYAYATGYTAGA
8R3	CCATCAAGACTGG	AACACTCGCAYAYATGYTAGA	CCATCAAGACTGGAACACTCGCAYAYATGYTAGA
8R4	CCATCACAAGGAC	AACACTCGCAYAYATGYTAGA	CCATCACAAGGACAACACTCGCAYAYATGYTAGA
8R5	CCATCACCTCACT	AACACTCGCAYAYATGYTAGA	CCATCACCTCACTAACACTCGCAYAYATGYTAGA
8R6	CCATCACGGAATG	AACACTCGCAYAYATGYTAGA	CCATCACGGAATGAACACTCGCAYAYATGYTAGA

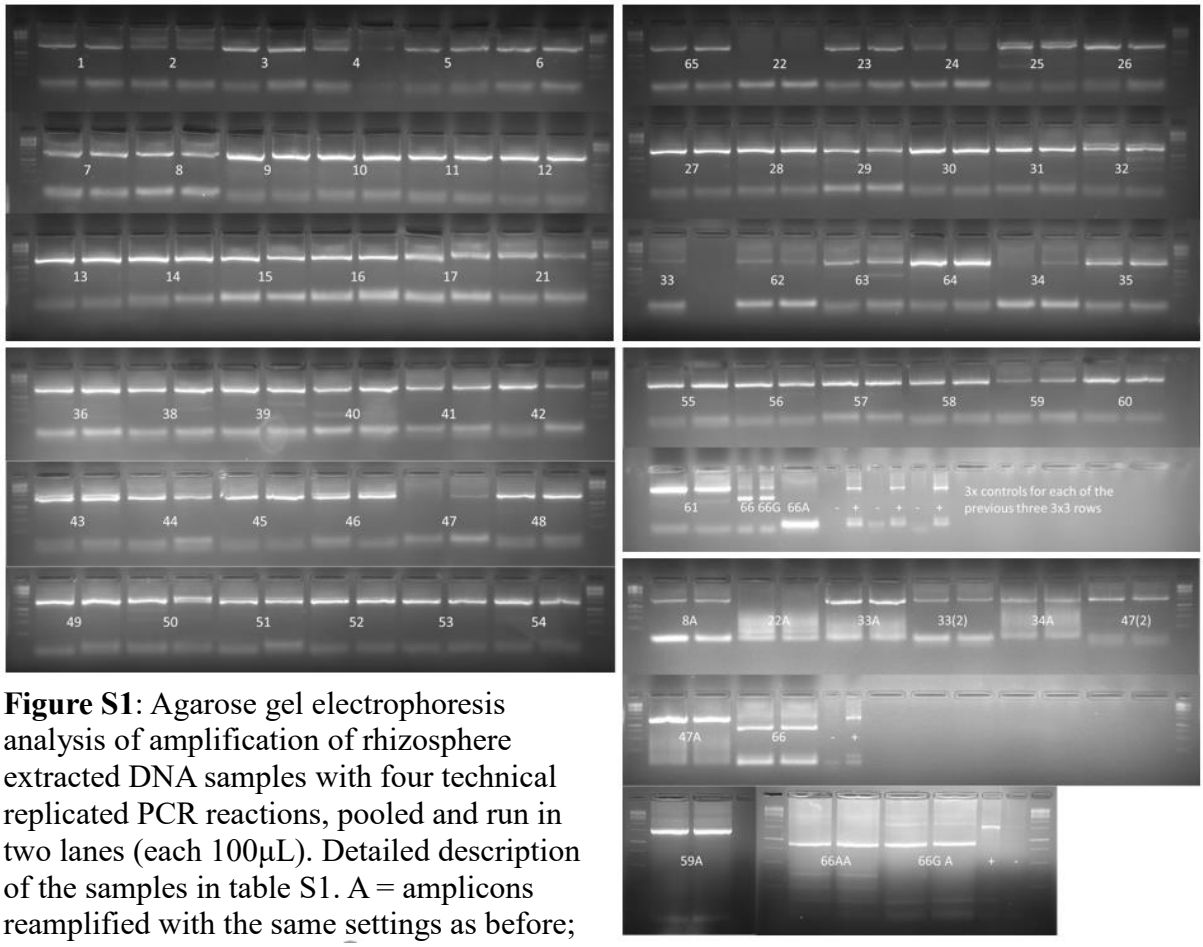

Figure S1: Agarose gel electrophoresis analysis of amplification of rhizosphere extracted DNA samples with four technical replicated PCR reactions, pooled and run in two lanes (each 100µL). Detailed description of the samples in table S1. A = amplicons reamplified with the same settings as before; G=original extract was amplified with the unspecific Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, PA, USA).

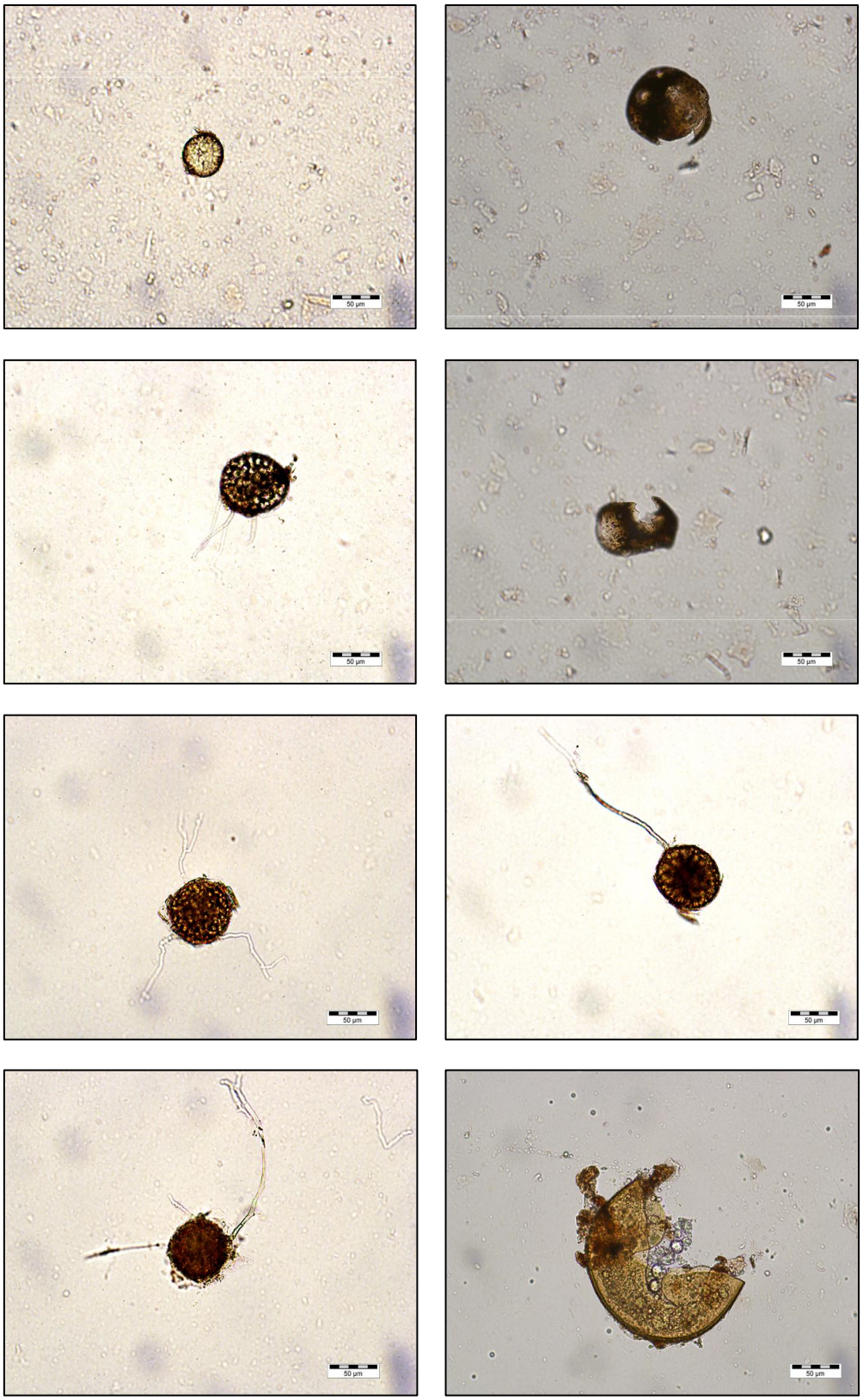

Figure S2: Microscope photos of the inocula. Top four photos show *Rhizophagus fasciculatus* and bottom four photos show *Glomus leptotichum*.

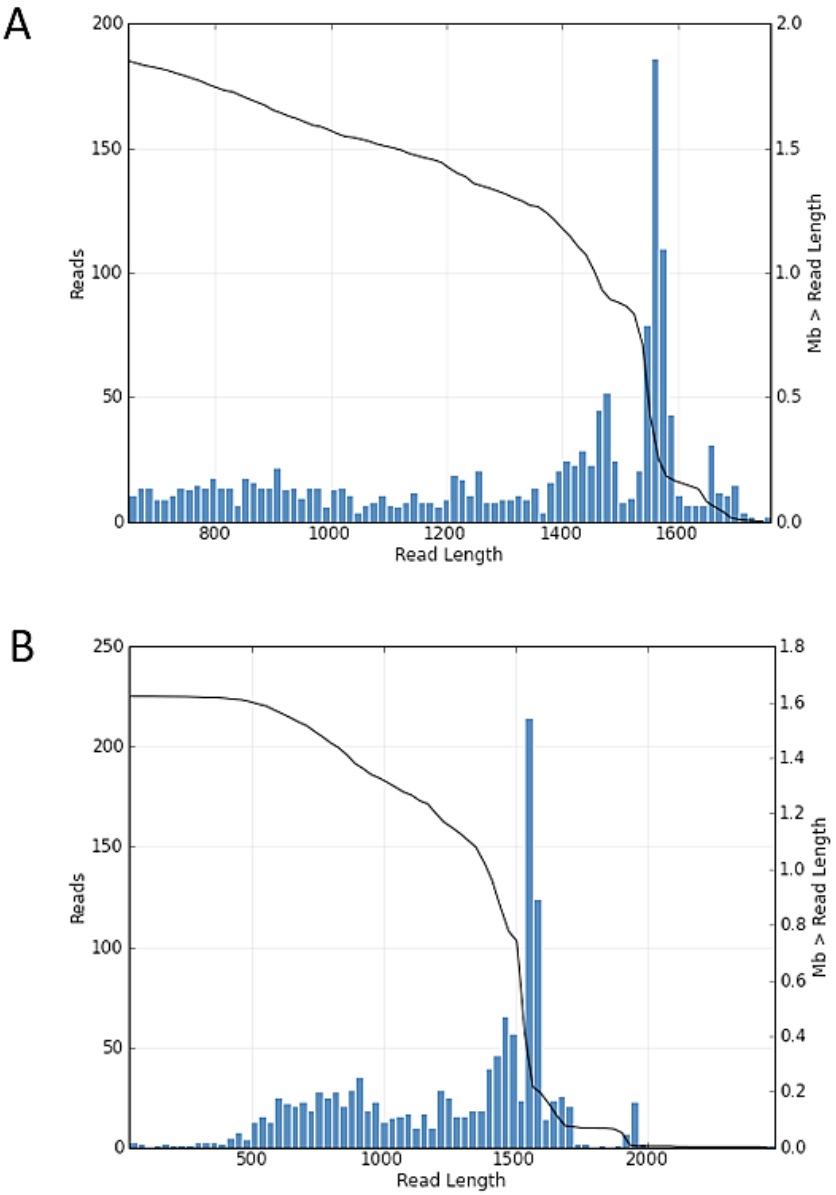

Figure S3: Read length of the obtained sequences from (A) filtered reads from 5CCS at 97% accuracy and (B) 1-pass reads at 99% accuracy.

The following study has been conducted by me, Lukas Schütz, and I am the main author. It is in preparation to be submitted to a peer reviewed journal jointly with other results about the community of bacteria and fungi assessed via culture methods in the lab.

Diversity of bacterial communities in the rhizosphere of a pigeon pea / finger millet intercropping system in India after application of biofertilizers, analyzed by automated ribosomal intergenic spacer analysis (ARISA)

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Abstract

Microbial population dynamics were examined in rhizosphere soil samples collected from pigeon pea and finger millet mono and intercropping. The study was conducted following the inoculation of AMF (*Rhizophagus fasciculatus* and *Glomus leptotichum*) and PGPR (*Pseudomonas fluorescens*), alone or combined, with mineral and organic fertilizers over two years at two different experimental sites in South India (GKVK, Bangalore, Karnataka and Kolli hills, Tamilnadu). Soil samples of 0 days and rhizosphere soil at harvest were collected from the field plots and soil of four field replicates was pooled. Automated ribosomal intergenic spacer analysis (ARISA) was conducted to find influencing factors of the bacterial community. We found clear differences between 0 days and at harvest when plants were present. Furthermore we identified crop species as the biggest driver of the bacterial community in the harvest samples. Differences were also found when each of the crops was grown in intercropping. No effect could be found for the biofertilizers. We conclude that biofertilization proved to be harmless to the rhizospheric bacterial community and their use poses neither a danger to delivered soil ecosystem services by the native bacterial community.

Keywords: biofertilizer, AMF, PGPR, ARISA, pigeon pea, finger millet, intercropping

Introduction

Mixed- or intercropping was the general practice in traditional agriculture and vanished only in the wake of the highly mechanized, industrial agriculture (Lithourgidis et al. 2011). Currently, however, intercropping comes of age again because of the urgent quest to find a more sustainable agriculture, using new technologies to obtain sufficient yields of good quality crops, also with respect to globally limited natural resources such as fertilizers (Bala et al. 2005; Cordell et al. 2009). This is particularly urgent in tropical and subtropical regions in the view of heat and drought stress induced by climate change (Battisti and Naylor 2009).

The application of microbial inoculants (biofertilizers) is an alternative method of increasing crop productivity that can reduce the use of chemical fertilizers. A wide variety of beneficial microbes interacts with plants and forms a broad spectrum of communities that differ between plants influencing their development and yield in various ways (Vessey 2003). Beneficial bacteria collectively known as plant growth-promoting rhizobacteria (PGPR) have a high potential to improve plant nutrient uptake, especially when they are applied in combination with AMF (Artursson et al. 2006). PGPR comprise different functional and taxonomic groups of bacteria such as *Pseudomonas*, *Bacillus*, *Rhizobia*, *Azospirillum*, and *Azotobacter* (Benizri et al. 2001; Ghosh et al. 2002). Their ability to fix atmospheric nitrogen or mobilize either minerally or organically bound nutrients from the pedosphere and make it available to the plants is highly interesting for agriculture. Furthermore, some stimulate plant growth directly by synthesizing plant hormones or indirectly by suppressing soil-borne pathogens, or by inducing plant resistance (Benizri et al. 2001).

Arbuscular mycorrhizal fungi are a dominant fungal association in the soil and in agricultural systems (Balakrishna et al. 2017) and support plant growth directly by enhancing nutrient (P, Zn and Cu) and water uptake (Bagyaraj 2014). AMF also interact with bacteria and mycorrhiza helper bacteria were shown to stimulate AMF spore germination, hyphal growth and AMF root colonization directly (Artursson et al. 2006; Frey-Klett et al. 2007). Conversely, AMF influence the chemical composition of root exudates, which in turn are a major nutrient source for the bacteria in the rhizosphere (Harris 2009). There is little knowledge on the effects of a combined application of such beneficial microorganisms on the indigenous microflora.

The present investigation was carried out to determine the effects of application of a consortium of AMF and PGPR alone or in combination with mineral fertilizer on soil microbial population

dynamics for sustainable pigeon pea and finger millet intercropping system performed over two years at two locations in India with different agro-climatic zones (GKVK, Bangalore, Karnataka and Kolli hills, Tamil Nadu). We used automated ribosomal intergenic spacer analysis (ARISA), a technique developed to "fingerprint" microbial communities (Fisher and Triplett 1999; Ranjard et al. 2001), in order to analyze the bacterial communities in the rhizosphere soil. Following question were addressed:

I. Can microbial inoculants or biofertilizers have an influence on the native microbial community?
II. Which factors and which management practices influence the microbial community?

Materials and Methods

Locations and experiments

The study sites were located at GKVK, Bangalore and Kolli hills, Tamilnadu. The experiments were performed in a plot with four field replicates per treatment in each location and in two consecutive years. Inoculation treatments were randomized in each replicate. Individual plot size was 6.6 x 3.9 m as a standard. Inoculants such as AMF and PGPR were inoculated and tested for plant growth response at two fertilizer levels in pigeon pea and finger millet mono cropping; pigeon pea-finger millet inter cropping systems at the two study sites. The plot size was 25 m^2 with total layout area of 2059.2 m^2. Low bands of soil were constructed between the plots to inhibit water flow and to minimize cross-contamination of the inoculants between the plots.

Inoculants and inoculation procedures

As bioinoculants, *Pseudomonas* sp. MSSRFD41 was used as a PGPR along with two AMF strains *Glomus leptotichum* (pigeon pea) and *Rhizophagus fasciculatus* (finger millet). Farm yard manure (FYM) was applied as blanket for all plots at 7.5 tonnes per hectare. The recommended dose fertilizer (RDF) was mixed in the ratio of 50–40–25 Kg NPK/hectare and 25–50–25 Kg NPK/hectare for finger millet and pigeon pea respectively and applied at 50 % RDF. The PGPR and AMF were mixed appropriately with vermiculite (carrier) and applied to pigeon pea, which was pre-grown in plastic containers, and to finger millet at the time of sowing.

Sampling

The treatments had been replicated from each of the two years field trial and the two sites Kolli Hills and Bangalore. Five randomly selected plants of each species in a plot were uprooted and the soil adhering to the roots was collected, thereafter referred to as rhizosphere soil. The soil was then pooled with the other three replicate plots. Thus each sample represents 4 pooled field replicates.

Soil DNA extraction

DNA was extracted from 250 mg of rhizosphere soil samples using the "power soil DNA kit" (MOBIO laboratories Inc., USA), following the manufacturers protocol, applying the bead beating and spin column filter extraction method. The soil sample added to the power bead tubes containing the suspension buffer (C1) and lysis buffer (C2) were added before vortexing at maximum speed, where lysis occurs by mechanical and chemical methods. Then the samples were treated with PCR inhibitor removing solution (C3) provided by the manufacturer to remove contaminants. The total DNA bound using binding solution (C4) to the silica membrane provided in the spin column is then washed (C5) and eluted with 50 µl of solution C6 from the membrane (www.mobio.com).

ARISA analysis

ARISA was used to study the genetic structure of the bacterial community in the rhizosphere samples. PCR was performed with primers appropriate for ARISA of bacterial communities (B-ARISA) to amplify the bacterial intergenic spacer located between the small- and large-subunit rRNA genes The primers used were: S-d-Bact-1522-b-S-20-FAM (5’ TGCGG CTGGATCCCCTCCTT 3’) and l-d-Bact-132-a-A-18 (5’ CCGGGT TTCCCCATTCGG 3’) (Ranjard et al. 2001).

PCR conditions were as follows: 95 ◦C for 15 s, followed by 15 cycles of touchdown protocol from 60 ◦C to 55 ◦C for 45 s, 25 cycles at 55 ◦C, and extension at 72 ◦C for 45 s. PCR products were tested on 1% agarose gel and then analyzed using the AB3130xl Sequencer. GeneScanTM 1200 LIZ® (Applied Biosystems, CA, USA) size standard was used to determine sizing up to 1200 bp. Raw data generated by the AB3130xl Sequencer were initially analyzed using PeakStudio (Mccafferty et al. 2012). Peaks were looked for between 150 and 700bp, and peaks were found between 239bp and 638bp. Peaks with less than 10 RFUs (relative fluorescence units) were excluded (Kovacs et al. 2010). No bins were formed and each peak was treated as a population. Each bin was thus considered an OTU (operational taxonomic unit). The data

were then exported to Microsoft Excel® for further analysis and converted to binary data, because the peak heights (in fluorescence units) were too variable to be used in further analysis. Binary data was prepared on the basis of presence or absence of the peak in the electrophoregrams. If a given OTU only appeared once in all treatments it was considered an artefact and excluded from further analysis. After cleaning the data in this way, species richness was determined by counting the number of OTUs in each sample (Kovacs et al. 2010). Further cluster analysis was conducted with R Software Version 3.2.3 and the interface R-Studio Version 0.99.491 using GGBIPLOT for graphical operations. The "prcomp" command was used to perform principal component analyses with the binary data. Peaks which appeared only once in the whole dataset were excluded from further analysis.

Results

ARISA of the bacterial community

A high diversity of OTUs was found at both sites (in total 218 at both sites for the first year and 271 at both sites for the second year field trial; after excluding unique OTUs at Kolli Hills 178 and at GKVK 179 in the first year and 224 and 243 in the second year). In the first year only 28 OTUs were shared between the two sites. In the second year 88 were shared OTUs. Thus a high level of "endemism" was found in both sites. The OTUs found in the second year samples was higher at both sites. With few exceptions (Kolli Hills: FM and PGPR, GKVK: PP with AMF and without inoculation), the highest diversity was found in intercropping for both plants in the samples from the first year. In the second year results showed less of a trend. Especially at Kolli Hills, the trend went in the opposite way, with a higher diversity under monocropping. Also at GKVK the diversity was higher for monocropped finger millet, but with pigeon pea under intercropping, the PGPR treated and noninoculated treatments showed a higher number of OTUs than the monocropped pigeon peas. For the inoculation no trend could be detected in the number of OTUs (Fig. 1).

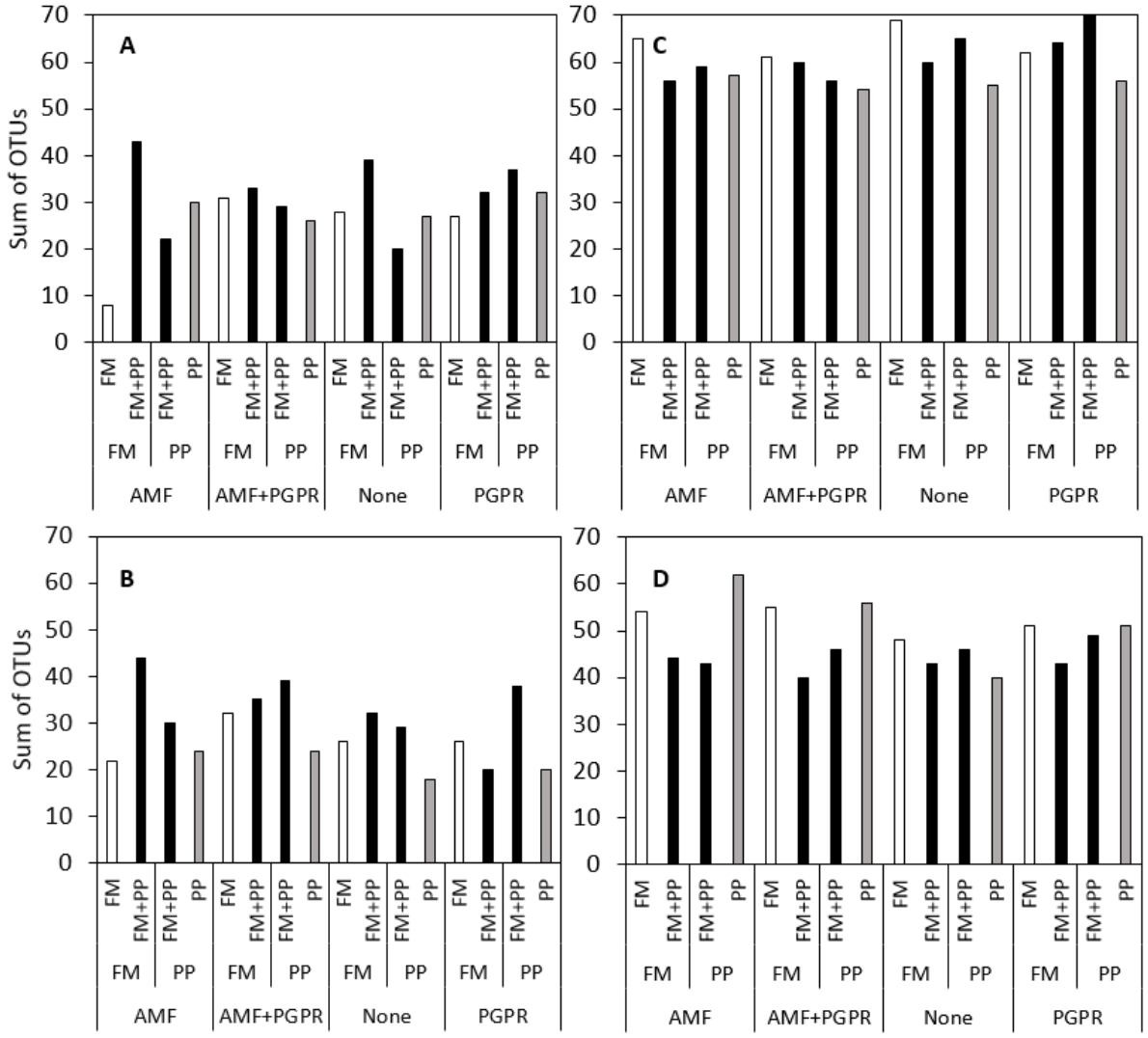

Figure 1: Diversity of bacterial communities in the first and second year of the field trials of GKVK (A, C) and Kolli Hills (B, D) rhizosphere soil based on the number of OTUs obtained from ARISA analysis.

A PCA allowed a deeper analysis of the differences between bacterial communities. Inoculation of either inoculant had no significant effect on the community composition at both sites and in both years of the field trials (Fig. 2). Bacterial diversity was rather determined by the crop and the cropping system. In the first year the bacterial communities of monocropped pigeon pea or finger millet showed no overlap in the PCA. Results from the second year confirmed this effect at GKVK site. Each intercropped crop had a larger ellipsoid assigned that the monocropped crop indicating a larger diversity in intercropping. Clear differences in the bacterial community diversity could be found between samples taken just before planting ("preplanting") and the samples taken at harvest time (Fig. 3). However the number of OTUs found before planting was low in the second year at GKVK site which on its own creates clear difference to the

sampling at harvest. At Kolli hills site almost no OTUs were found in the soil of the intercropped plots before planting (Fig S1).

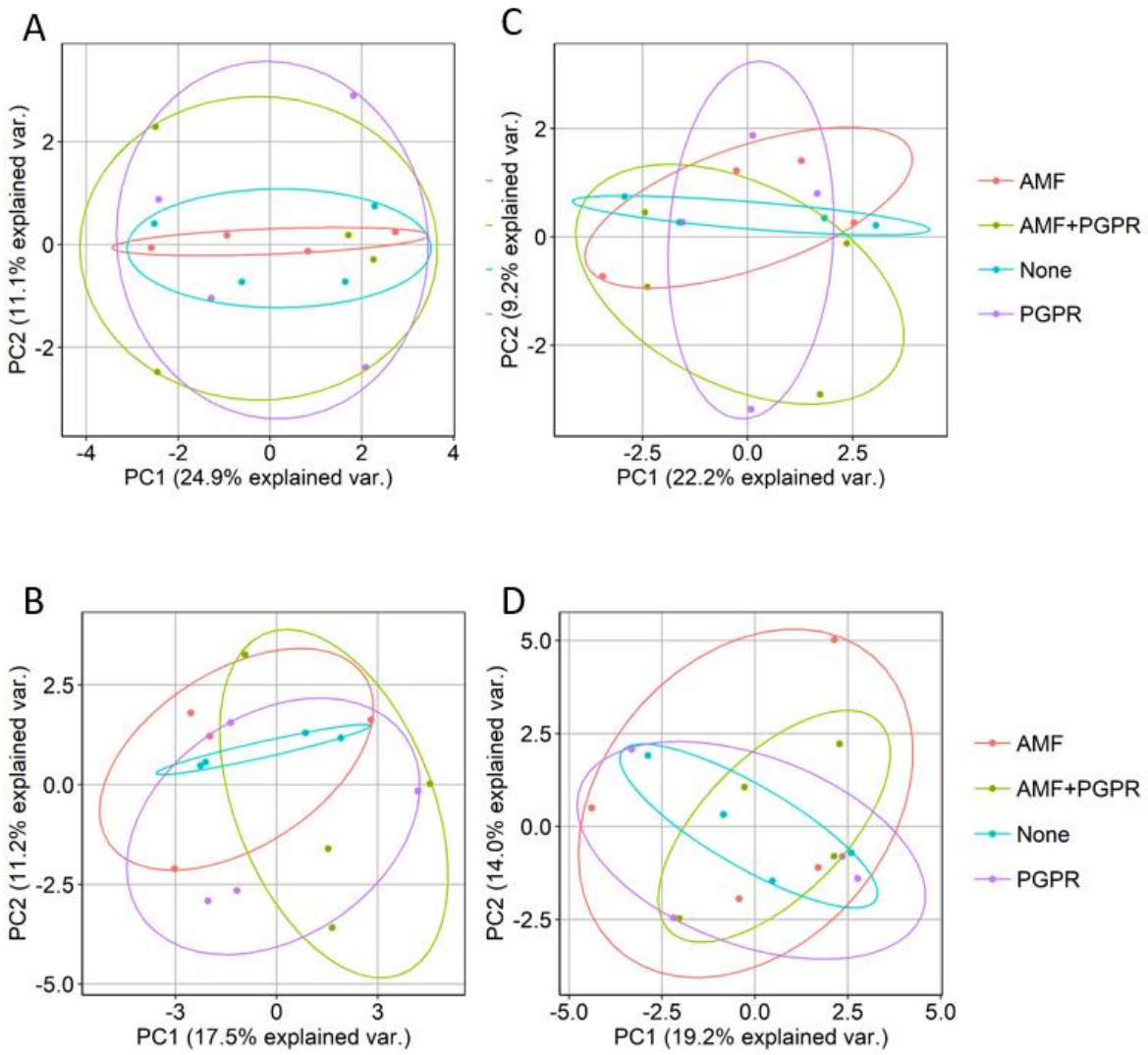

Figure 2: Principal component analysis of the ARISA data on the diversity of bacterial communities, as affected by bioinoculants. When circles overlap there is no significant difference between groups. A and B show the results from first and second year at GKVK and C and D the results from first and second year at Kolli hills.

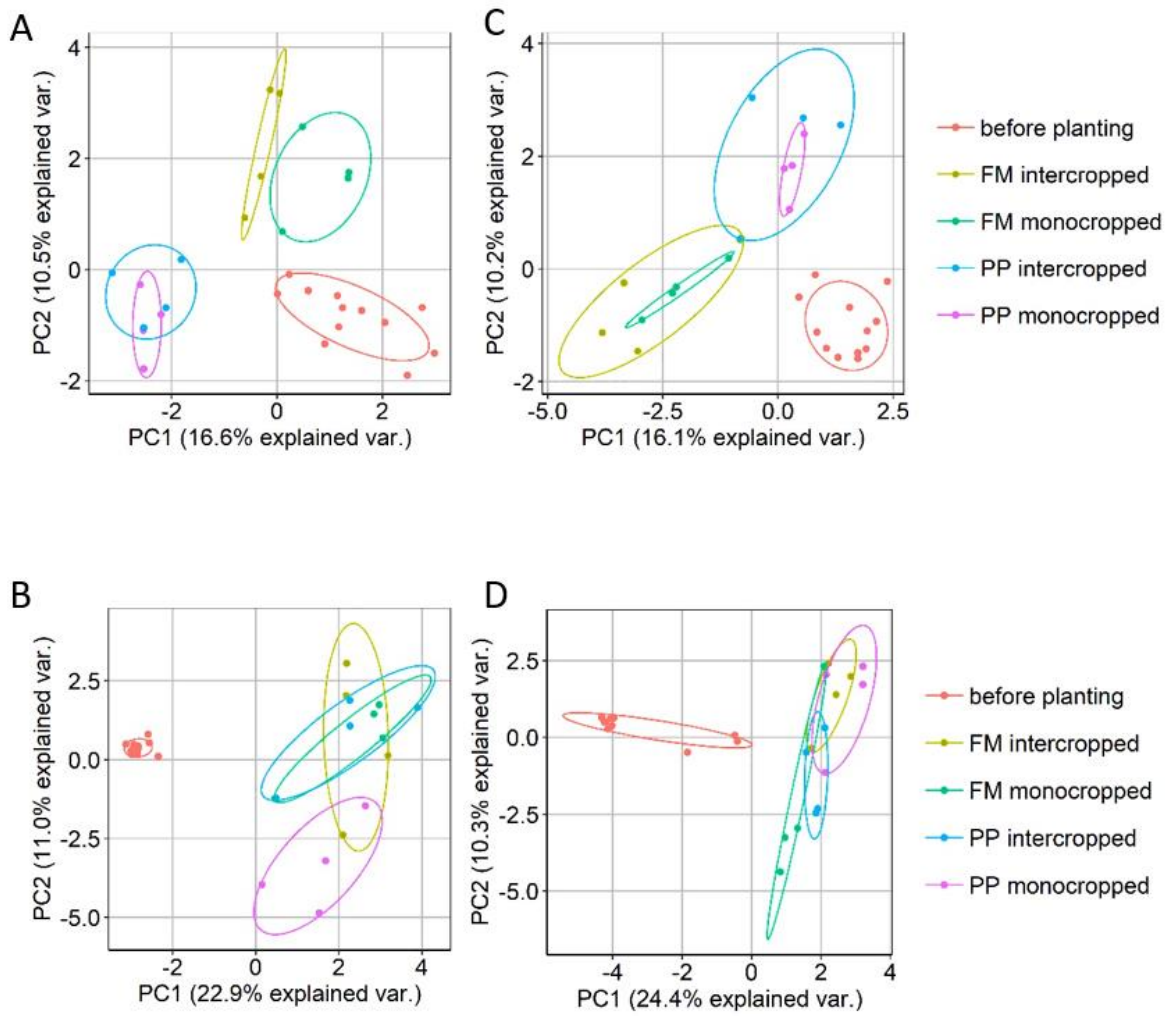

Figure 3: Principal component analysis of the ARISA data on the diversity of bacterial communities, as affected by the cropping system and compared to the data for preplanting. When circles overlap there is no significant difference between groups. A and B show the results from first and second year at GKVK and C and D the results from first and second year at Kolli hills.

Discussion

ARISA is a simple and inexpensive method to characterize bacterial diversity, but its results have been found to be similar to the ones provided by next-generation sequencing (Gobet et al. 2014; van Dorst et al. 2014). (Note that none of these techniques assesses the functional diversity.) Using ARISA, we have detected no changes in the diversity of the bacterial community upon the inoculation of AMF and PGPR indicating that the inoculation with bio-inoculants did not visibly disturb the microflora in the soil.

Other factors, such as the plant identity and whether it was grown alone or in intercropping, had a larger influence on the diversity of the bacterial community. The largest difference was between the microbial diversity at planting time and at harvest time. These differences were very convincing in three of the comparisons undertaken, but were based on data with high variability at Kolli hills in the second year. This variability is being accounted for in the PCA but we cannot exclude a handling error during the sampling for the intercropping plots. Interestingly we also detected a larger diversity when each of the crop was intercropped. Also here it is not possible to conclude anything about functionality, but having a higher diversity in the cropping system is causing a higher bacterial diversity underground. This would increase the chances to have more functionality and a better delivery of ecosystem services because a higher diversity is in theory also connected to more functionality with a higher diversity in enzymes, carbon sources etc. (Mace et al. 2012).

Agriculture creates big changes underground by tilling, fertilization or crop rotation. The exact changes through the application of a biofertilizer are actually difficult to find. Also whether microbial inoculants can change soil ecosystem services at all and over which time is an unresolved question. There is a need to have replicates to know about the variation in a sample and to make sound conclusions. But then microbial communities differ on a very small scale (Torsvik and Øvreås 2002) which makes it difficult to sample the same microbial community and find the effect by the management practices during a scientific study.

Despite these aspects about the resolution we were able to identify crop species as well as the cropping system to influence the bacterial community. Furthermore despite the worries about biofertilizers changing the soil microbial community we cannot confirm this and our results are evidence for the harmlessness of biofertilizers in this aspect.

Supplement

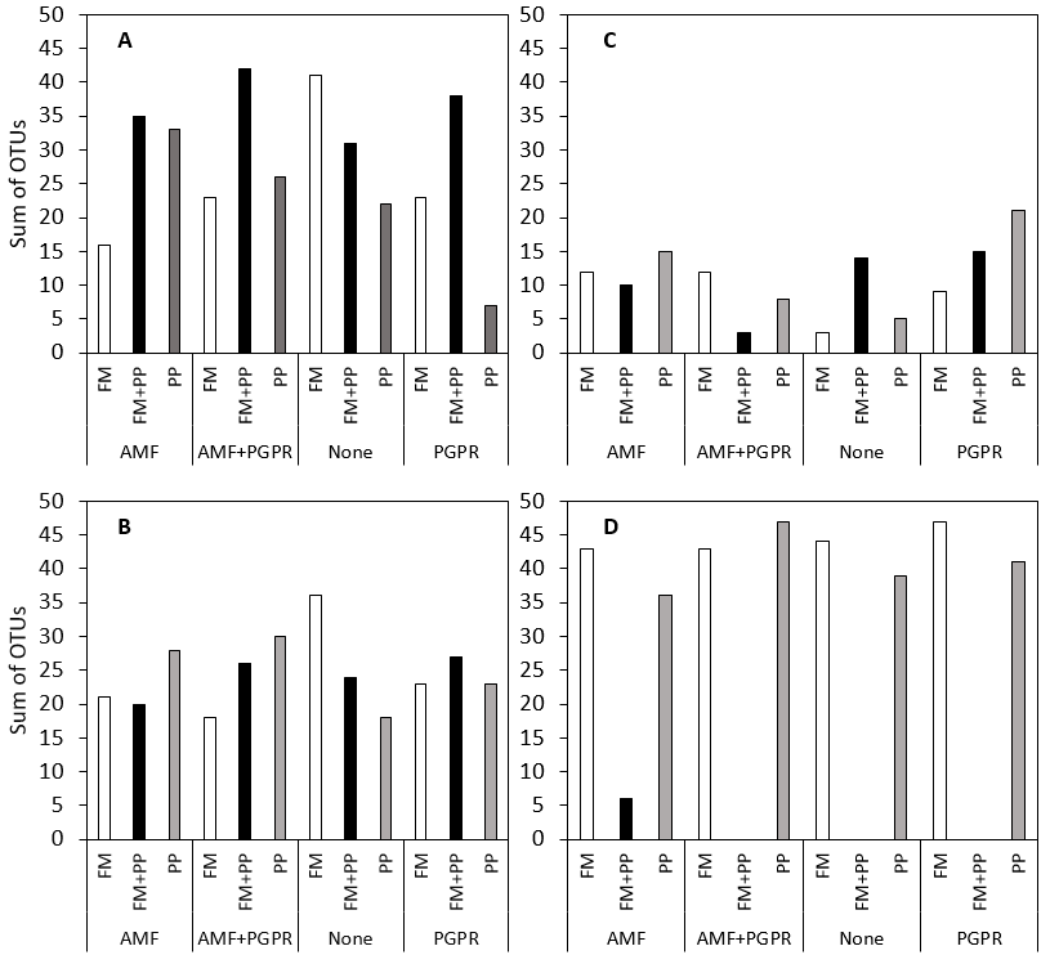

Figure S1: Bacterial population dynamics in the first and second year of the field trials of GKVK (A, C) and Kolli Hills (B, D) soil from the plots before planting based on the OTUs obtained from ARISA analysis.

General discussion

Worldwide application of biofertilizers

Our meta-analysis provides new insights how biofertilizers can be applied in a more directed and systematic way. The most promising geographical areas are in the tropics and subtropics and also in arid and semi-arid regions of the world. In humid continental and oceanic climates the application did also increase yields but to a smaller extent. Thus for the previous group the application of biofertilizer can be clearly recommended. Yield increases there are in a size order as high as or higher than other future technologies in agriculture. This could be consolidated by higher success under rainfed conditions and the dependency of certain biofertilizer groups to soil available P. The parameter yield was the focus of the meta-analysis because it is easy to calculate and to understand. Future technologies need to also focus on sustainability aspects and make more efficient use of the natural resources. And also here biofertilizers are very promising and improve the nutrient use efficiencies of P and N as shown by our study.

Rosegrant et al. (2014) have compared 11 different modern agricultural technologies and tried to model their promise to increase the yield of major crop plants until 2050: no-till; integrated soil fertility management (ISFM); precision agriculture (PA); organic agriculture (OA); nitrogen-use efficiency (NUE); water harvesting; drip irrigation; sprinkler irrigation; improved varieties-drought-tolerant characters; improved varieties-heat-tolerant characters; crop protection. Surprisingly, they did not look at biofertilization. However, their projections are interesting when compared to our analysis. (Note that data generated by such models for the future are difficult to compare because of the different baselines used in the calculations. If the baseline is the current practice of one region or country it will differ from place to place. If the baseline is standardized it may not be suitable for all regions.)

Rosegrant at al. have modelled the yield gains until 2050 of maize, rice and wheat with their Decision Support System for Agrotechnology Transfer (DSSAT) crop model taking into account spatial variability of crop production, climate, soil, and projected climate change which gives the most realistic data and can be compared to our data. No-till farming are projected to increase maize yields by 20%. Yet together with sufficient irrigation these maize yields could increase up to 67% (Rosegrant et al. 2014). Similar results were calculated for wheat. Improving the nitrogen use efficiency, a mixture of timing of nitrogen application and breeding, was most efficient for rice (22% rainfed & 43% irrigated), but also for maize under irrigated conditions (52%). Increased heat tolerance improved yields greatly in maize (31% rainfed & 37% irrigated) and wheat (16% rainfed & 28% irrigated). Precision agriculture ranked among the

best technologies for wheat (25% rainfed & 30% irrigated). To increase food security and improve productivity and sustainability the optimal solution will be a combination of these technologies, modelled as well by Rosegrant et al. (2014). Different sets of technologies will be appropriate in different regions of the world, and depend on the availability of certain technologies, the training or susceptibility to climate change. For developing countries this could be no-till farming, nitrogen-use efficiency, heat-tolerant crops, and crop protection from weeds, insects, and diseases (Rosegrant et al. 2014).

The results of our meta-analysis describe the actual state and do not have the long-term perspective as the modelled results from the above study. Yet the data are promising, and biofertilizers are definitely one of these future technologies under the right pedo-climatic conditions and when the right biofertilizers are selected. Especially the results from dry climates under rainfed conditions are interesting and make them a future technologies when a higher resilience to drought conditions and climate variability is needed. Apparently they can close a gap in the recolonization of the soil by microbes after dry soil is wetted again. After rain or irrigation also nutrients are released from mineralization and by microbial biomass which die from the osmotic shock after getting in contact with water. The facilitation of the uptake of these nutrients may be another way how they improve the establishment of crops.

Interestingly facilitation by N fixers was indifferent to increased P levels in the soil due to P fertilization. Observations from my meta-analysis not published here showed that also increased levels of fertilized N had no negative effects on the yield promotion of P solubilizers. Hence it may be possible to increase yields of crops already well supplied with nutrients which would make them a unique case within the above discussed technologies.

Also the ratio of work and money that has to be invested to the expected outcome will be highly important on the adoption success of modern agricultural technologies. Biofertilizers are applied in small quantities, and the requirements for labour in the field do not increase a lot. However farmers need to be trained how to use them and have access to an extension service which can advise them on which biofertilizer works best for their region and crop. Additionally the biofertilizers have to be cultured and multiplied for the application. This can be done on site provided there is initial training available, as pioneered by the MS Swaminathan Research Foundation in Chennai (http://www.mssrf.org). Alternatively, the biofertilizer are produced in an external lab or company. This requires the transport to farmers in more remote areas and

sufficient storage time and survival of the biofertilizers. The low cost of production makes the application of biofertilizers already profitable with low increases in yields and thus income. Hence also the risk of buying biofertilizers is low and even a failure would not be as costly as an irrigation system for example.

The success of the inoculation with biofertilizers could be reduced by an increased use by some of the above technologies (Rosegrant et al. 2014). Our own analysis showed that the external application of biofertilizers is less successful in the case of AMF and less strong but also for P solubilizers and in combination with N fixers being less effective at promoting yield under high content of organic matter. Other studies also came to the conclusion that organic matter is important for an active, abundant, diverse and adaptive microbial community and support our findings (Emmerling et al. 2002; van der Heijden et al. 2008; Nielsen et al. 2015; Lori et al. 2017). Organic matter is often the target to improve soil fertility and soil functioning e.g. in the above discussed integrated soil fertility management, no-till agriculture and organic agriculture. Organic matter can capture nutrients and make them available over a longer time period and prevent leaching (Magdoff and Weil 2004). It can also sequester carbon during its formation and reduce greenhouse gas emissions (Paustian et al. 1997; Snyder et al. 2009). It is also known to increase the water storage of soils and increasing soil stability thereby reducing erosion (Lynch and Bragg 1985; Rillig and Mummey 2006). Thus sustaining organic matter, or increasing it, is highly desirable for several reasons but will reduce the success of AMF inoculants.

Having a more fertile soil with higher water holding capacity, higher organic matter content, more soil stability and overall a higher resilience can only be achieved with a different farming system that applies enough organic matter like in organic farming or conserves the soil like in no-tillage farming. Biofertilizers cannot fulfill all the functions that a whole system like organic farming in combination with some of the above mentioned technologies can fulfill. Thus their application cannot replace a more thorough change of the system when aiming at improving sustainability in agriculture. Yet they can support these aims. The easiness of applying biofertilizers make them a much quicker solution of improving yields than some of the approaches discussed by Rosegrant (2014), and it could be applied in combination with most of them . In this regard, biofertilizers may have various different functional traits, such as the ability to fix atmospheric N, to access nutrients such as P and N from organic fertilizers, and to improve drought tolerance and to promote plant health.

Selection of the optimal biofertilizer strains

Biofertilizers differ in various aspects of their functionality and their competitive traits which influence their suitability as biofertilizers for a certain crop, cropping system or soil. Many times biofertilizers are selected in the lab first before they are tested *in situ* to identify the most promising one. They are tested for their ability to solubilize nutrients from immobile forms like P from $FePO_4$, reduce nitrate, oxidize Sulphur, fix nitrogen, produce plant hormones like IAA, produce ACC, a precursor of the plant hormone ethylene, or whether they can form spores which is important their survival and storage time of a commercial product (Shaharoona et al. 2007; Omer 2010; Salimpour et al. 2012; Arora 2013; Herrmann and Lesueur 2013). To predict their competitive behavior and survival after application microorganisms are tested for their production of antibiotics, siderophores, HCN production or whether they can form biofilms (Compant et al. 2010). Furthermore it is desirable for the inoculants to firmly establish and survive over several years to benefit from the growth promotion over longer time without having to apply them every cropping season.

We have tested different AMF species for their ability to promote growth and yield of finger millet, to promote growth of pigeon pea, their ability to transport nitrogen, their ability to spread through bare soil without the presence of roots over different distances and their rate of hyphal spread. We found different species being more suited when the distance between the plants is short (*R. fasciculatus*) or long (*C. etunicatum*). One species (*R. irregularis*) promoted the growth of pigeon pea, had fast growing and exploring hyphae, but seemed to be rather less beneficial to finger millet since it did not transport nitrogen to finger millet. The decision for which species will be best in a field application is difficult to answer with these results. However such results can only be recorded in the lab and greenhouse and are important to understand the functioning of mycorrhizal symbiosis in a an intercropping system where pigeon pea (*Cajanus cajan*) seedlings are pre-inoculated with AMF and planted into a field sown with finger millet (*Eleusine coracana*). Microorganisms are often overlooked in an intercropping system. In the field the density and distance between plants has been shown to be crucial for the success of an intercropping system (Dhima et al. 2013) and also for pigeon pea - finger millet intercropping systems (Padhi et al. 2010). One reason for this could also be the benefit of a CMN, such as studied in this thesis.

We have recorded the fastest hyphal spread ever measured for AMF. Depending on the row distance one can estimate when the roots of the plants will be connected via mycorrhizal hyphae. The distance in the field trial between the pregrown pigeon pea and the center of the

finger millet row is about 20cm which the hyphae of *C. etunicatum* would cover in about seven weeks. Therefore our initial hypothesis that the pre-inoculation of pigeon pea with AMF will be enough to lead to colonization of neighboring rows of finger millet has to be rejected.

Biofertilization and microbial community changes

In our field experiment, we could not precisely trace the AMF and PGPR species applied as biofertilizers, although in both years and sites the grain and straw yields of pigeon pea and finger millet at 50% mineral fertilization were significantly increased compared to the uninoculated control, which provides indirect proof of their beneficial effects (Mathimaran et al., in preparation). We also studied the community composition of indigenous bacteria and AMF, and I discuss some of these results below.

The initial population with AMF measured with spore concentration in soils of the field sites ranged from 77 to 233 spores/100g in 0-15cm depth in Bangalore field site and 80 to 133 in Kolli Hills field site (Muthukumar, personal communication). Hence the inoculum of the two applied AMF species might have faced competition from the native AMF community. Nevertheless the deposition of the AMF inoculum in close vicinity of the germinating seeds may give the AMF biofertilizer a certain advantage over the indigenous AMF spores germinating further away. Still the analysis of the AMF community in the rhizosphere, based on rDNA sequencing, shows that in both years of the study, finger millet roots were surrounded also by various native AMF species. AMF DNA from the roots could not be amplified sufficiently so direct conclusion of the root colonization is not possible, because the DNA extracted from the rhizosphere could also contain DNA from resting spores.

How exactly the growth promotion by applying AMF inoculum is achieved is governed by several factors and depends on the native mycorrhizal community. The reason for a growth promotion under competition of different AMF species are probably complementary effects with AMF differing in their life traits or nutrient foraging strategies (Koide 2000), hence leading to a better growth promotion with higher diversity of AMF. But under competition the growth promotion of the plant can also be reduced (Janoušková et al. 2013). Werner and Kiers (2015) studied priority effects in a model system with two AMF species. They and other studies (Pearson et al. 1993; Vierheilig et al. 2000; Vierheilig 2004) found that the invading species was suppressed by the indigenous species depending on the time lag between inoculations. The authors in the above studies conclude that either available root space is quickly occupied by the early colonizers or the plant/AMF may suppress subsequent colonizers. AMF species can

physically block each other's colonization, as shown by using spatially separated inocula (Hepper et al. 1988). Yet also the host may be able to regulate their carbon allocation to different mycorrhizal partners (Kiers et al. 2011). These interesting results illustrate the complexity of plant-AMF and AMF-AMF interactions, but do not lend themselves to generalizations.

Once the microbial inoculants are established, they have an effect on the microbial community and possibly also the soil. Whether microbial inoculants can change soil ecosystem services and over which time-scale is an unresolved question. If they establish or become invasive and propagate into neighboring soil ecosystems any changes in the service provision would be irreversible. Through ARISA we have detected no changes in the bacterial community by the inoculation of AMF and PGPR. ARISA is a "fingerprinting" method to measure and compare the diversity of microbial communities; it does not assess the functioning of the microbial community, thus we cannot conclude anything in depth. However, the overall results of ARISA analysis and next-generation sequencing of ribosomal DNA markers are similar (Gobet et al. 2014; van Dorst et al. 2014). Our ARISA analysis did not reveal significant differences between the different biofertilization treatments. However, it clearly showed a change in the microbial community for the different crops and for the beginning to the end of the planting season.

Outlook

My results show that biofertilization can increase yield in agriculture substantially, while at the same time avoiding undesirable side effects. However, there are problems when trying to transfer these results to the local level mostly because of the large diversity in soil microbes which biofertilizers interact with. But still my results will give biofertilizer programmes a new frame because the here identified abiotic factors and dependencies of certain groups of biofertilizers on these allow to narrow down regions and soil conditions under which the application of biofertilizers is promising. In the future, pretests of the soil community may also predict the competitive chance of biofertilizer. These tests might be based on high-throughput sequencing which already helps to improve the mechanistic understanding between microbial diversity and ecosystem functioning (Zhou et al. 2015).

Yet agriculture is not only natural sciences but has a social side too. Poverty is higher in rural areas (Rapsomanikis 2015) and especially the rural youths migrate to the cities in search of better jobs and apparently quit their farming practices learnt for generations. Currently, smallholder farmers produce 80% of the world food supplies (Food and Agriculture Organization of the United Nations 2014). To sustain crop production and food security, either

these farmers need to receive more support in form of extension services to develop a diversified, economically viable cropping system and to improve their living conditions, or agriculture will have to adopt more technical solutions and increase farm size with less work force.

With enough awareness for the big global challenges of climate change and an ever increasing world population, both scenarios may lead to a sustainable and resilient agriculture and food production. But a system which modernizes traditional farming methods with a so-called ecological intensification is expected to produce more diverse food from diverse adapted varieties while at the same time ensuring the delivery of the necessary agricultural ecosystem services (Bommarco et al. 2013). One example is the here studied intercropping systems with pigeon pea and finger millet inoculated with biofertilizers which adopts the traditional mixed cropping of South India and introduces row planting for the possibility to use farm machinery for the harvest and combines it with the application of biofertilizers. Hence the existing knowledge among traditional farmers about the local environment and adapted crops needs to be merged with scientists and policy makers to create site specific solutions as cropping systems cannot be generalized due to the large spatial variations in pedo-climatic conditions (Altieri 2004).

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